Intraspecific variation in defence and recovery of *Eucalyptus globulus* from mammalian herbivory

Christina Lilly Borzak

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Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Christina Lilly Borzak

Statement of co-authorship

The following people contributed to the publication of work undertaken as part of this thesis:

Christina L. Borzak, School of Biological Sciences, University of Tasmania, Hobart, Australia = **Candidate**

Julianne M. O'Reilly-Wapstra, School of Biological Sciences, University of Tasmania, Hobart, Australia = **Author A + supervisor**

Brad M. Potts, School of Biological Sciences, University of Tasmania, Hobart, Australia = **Author B + supervisor**

Karen M. Barry, School of Agriculture Sciences, University of Tasmania, Hobart, Australia = **Author C + supervisor**

Noel W. Davies, Central Science Laboratory, University of Tasmania, Hobart, Australia = **Author D**

Elizabeth A. Pinkard, CSIRO Ecosystem Sciences and Climate Adaptation Flagship, Hobart, Austraia = **Author E**

Below are the details of which papers the co-authors contributed to, the contributions they made and where the papers can be found in the thesis:

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Candidate was the primary author, and undertook all glasshouse work and sample preparation for terpene extraction. The Candidate as well as authors A and B contributed to developing the conceptual framework, design and approach. Author D undertook the GC-MS analysis of the foliar terpene content. Authors A and B assisted with refining the manuscript.

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Candidate was the primary author, and undertook all glasshouse and nursery work, morphologic assessments and data analysis. The Candidate as well as authors A-C and E contributed to developing the conceptual framework, design and approach. Author C assisted with photosynthetic rate and chlorophyll content assessments. Authors A-C assisted with refining the manuscript.

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Candidate was the primary author, and was involved in field work and undertook most of the analysis. The Candidate as well as authors A and B contributed to developing the conceptual framework, design and approach. Author B assisted with data analysis. Authors A and B assisted with refining the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____

Julianne M. O'Reilly-Wapstra

Supervisor

School of Biological Sciences

University of Tasmania

Date: 2 November 2015

Signed: _____

Anthony Koutoulis

Head of School

School of Biological Sciences

University of Tasmania

Date: 2 November 2015

Abstract

The interaction between plants and their herbivores has played an important role in driving the form of many plant traits, including defensive chemistry and plant recovery following loss of biomass. Identifying intraspecific variation in such traits is not only crucial for our ecological and evolutionary understanding of plant-herbivore associations, but also for how they can shape the structure of natural ecosystems. In a series of glasshouse, nursery and field trials I explored plant-mammal associations by investigating intraspecific variation in foliar chemical defence and plant recovery after damage (including physiological and morphological traits) in early life stage *Eucalyptus globulus*. The key research questions were 1). Is there ontogenetic variation in foliar terpenes during early stage development of *E. globulus* seedlings, and does this vary at the population and family level? 2). Is there intraspecific genetic variation in photosynthetic rate, chlorophyll content, defensive chemistry and growth response to partial artificial browsing of juvenile *E. globulus*. 3). Is there intraspecific genetic variation in *E. globulus* seedling survival and growth from lignotubers after severe artificial browsing. 4). What is the pattern (spatial and genetic) and direct impact of natural mammalian browsing on *E. globulus* seedlings in the field, and subsequent indirect effects on a dependent foliar organism.

A comparison of different populations of *E. globulus* showed significant genetic and ontogenetic variation in foliar terpenes throughout seedling development with a series of both dynamic and stable responses. Ontogenetic trajectories differed among populations suggesting that evolution may exploit the ontogenetic patterns of change in these compounds to create a diverse chemical mosaic with which to defend the plant. A key finding was the different behaviour of the two major terpene groups, which reflects their biosynthetic origins. Sesquiterpenes changed more rapidly throughout early ontogeny compared to monoterpenes, with sesquiterpenes expressing opposite trajectories between compounds.

Despite the large constitutive differences that exist in foliar chemistry and storage allocation between *E. globulus* populations, defoliation elicited conservative

recovery responses in physiological, growth and foliar chemical traits. Populations subject to a partial defoliation treatment (50% leaf removal) initiated regrowth from axillary buds. The remaining leaves on treatment plants showed dramatic increases in photosynthetic rates and chlorophyll content, whilst there was increased allocation to regrowth biomass as well as a change in leaf structure to increase light capture. A number of foliar chemical compounds showed elevated levels in the regrowth arising from axillary buds compared with new growth derived from apical meristems. In another trial, severe defoliation (decapitation) had clear effects on plant morphology, but after 20 months of growth in a field trial, the treatment effects on above-ground growth were overridden by population differences. There was, however, evidence of genetic divergence among populations in survival after severe defoliation. Survival among populations was different after a decapitation treatment, whereby the population with increased survival had relatively lower concentrations of foliar chemistry and better developed lignotubers (dormant buds). This result suggests that resource allocation to storage structures has been responsive to selection, and affects the ability of plants to recover from severe defoliation.

The survival and growth consequence of browsing by free ranging marsupials was assessed in a large scale genetic pedigreed field trial. While no genetic differences between trees in browsing damage were detected, damaged trees suffered increased mortality and reduced growth (assessed up to 4 years). Herbivore-induced changes in plant morphology also affected subsequent tree use by an invertebrate herbivore. This demonstrates the potential role that mammals could play in driving ecological communities, and provides new evidence of plant mediated indirect effects structuring dependent ecological communities in a eucalypt system.

The work presented in this thesis demonstrates the complexity and dynamics of the different strategies eucalypts use to deal with herbivore interactions. Mammal browsing damage had important direct effects, but there was genetic stability in many chemical and plant recovery responses, with changes in some traits potentially having flow-on consequences on other herbivores. In addition to ecological and evolutionary relevance, these results have important applied

implications for the development of management strategies by enabling prediction of tree productivity after a browsing event, and the implementation of herbivore strategies in managed systems, such as the use of genotypes with improved recovery.

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List of publications and presentations arising from this thesis

Refereed journal articles

Borzak, CL, O'Reilly-Wapstra, JM, Potts, BM. (2015a). Direct and indirect effects of marsupial browsing on a foundation tree species. Oikos **124**:515-524.

Borzak, CL, Potts, BM, Davies, NW, O'Reilly-Wapstra, JM. (2015b). Population divergence in the ontogenetic trajectories of foliar terpenes of a *Eucalyptus* species. Annals of Botany **115**:159-170.

Conference presentations

Borzak, CL and O'Reilly-Wapstra, JM and Potts, BM, 'Indirect and direct plant-herbivore interactions in a eucalypt system', 33rd Ecological Society of Australia Annual Conference Scientific Program, 1-5 Dec, Sydney, Australia, pp. 64. (2008).

Borzak, CL, O'Reilly-Wapstra, JM, Barry, KM, Pinkard, EA and Potts, BM. 'Investigating a genetic basis to physiological responses of *Eucalyptus globulus* seedlings to defoliation'. Speed talk and poster presentation at the XVIII International Botanical Congress, 23-30th July, Melbourne, Australia. (2011) [Conference Extract P0466].

Borzak, CL, O'Reilly-Wapstra, JM and Potts, BM, 'Investigating early ontogenetic development of foliar chemical defence in *Eucalyptus globulus*'. Speed presentation at the Ecological Society of Australia Annual Conference Scientific Program, 21-25 Nov, Hobart, Australia, pp.105. (2011).

Borzak, CL., Potts, BM., Barry, KM., Pinkard, EA. and O'Reilly-Wapstra, JM. 'Genetic stability of physiological and plant secondary metabolite responses to defoliation in a Eucalypt'. Poster presented by J. O'Reilly-Wapstra at the 12th Gordon Research Conference on Plant-Herbivore Interactions, 24 Feb-1 March, Ventura, California U.S.A. (2013).

Format of thesis chapters

My thesis consists of six chapters. Two of these chapters (chapters 2 and 5) have been published as peer-reviewed articles in international journals (Borzak *et al.* 2015a; Borzak *et al.* 2015b). The remaining two experimental chapters are presented as manuscripts ready to submit for publication. Being paper style means that some repetition of concepts and ideas was unavoidable, particularly in the introduction sections. The text in the published papers has been reformatted, and the references have been consolidated into a single section in the thesis. Chapter 1, the general introduction, provides the background to the thesis and also explains the thesis aims and structure. Chapter 6 synthesises the experimental work and summarises the key findings of the study and advances achieved in the field of plant-herbivore interactions in a eucalypt system.

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Chapter 1:

General Introduction: Plant defence and recovery to mammal browsing in a eucalypt system

1.1 Plant-herbivore interactions

Over the lifetime of a plant, it will interact with a multitude of species that can have negative effects on its fitness. Interactions between plants and their herbivores are among the most widespread species interactions, with important evolutionary consequences as herbivores influence plant structure and composition (Futuyma and Agrawal 2009). Loss of biomass to herbivores causes significant reduction in plant survival, growth and reproduction (Pisanu *et al.* 2012; Muiruri *et al.* 2015; Stephens and Westoby 2015) which is detrimental to plant fitness. Through selective pressure, plants have evolved a variety of mechanisms to cope with herbivore damage, and such interactions often involve recovery responses such as changes in physiology, morphology and foliar chemistry (Karban and Baldwin 1997). These changes may alter subsequent consumer communities and play a considerable role in shaping natural ecosystems (Ohgushi 2005; Genung *et al.* 2011; Ohgushi 2012; Hrabar and Du Toit 2014; Stam *et al.* 2014). Interactions involving foundation species, such as trees, may have particularly important implications as focal or keystone species, may structure communities by creating locally stable conditions for other species and by modulating and stabilizing ecosystem processes (Whitham *et al.* 2006).

To mitigate the negative effects of herbivore damage, plants have evolved strategies to defend themselves from attack. Plant chemical resistance is an important defence in many systems (reviewed in Mithöfer and Boland 2012) and there may be large inter- and intraspecific variation in the amount and type of chemical compounds employed (Kursar and Coley 2003; Andrew *et al.* 2007a; Gols *et al.* 2008; Holeski *et al.* 2012; Lindroth and St. Clair 2013; Moreira *et al.* 2013). Such variation prompts questions about the biotic (e.g. herbivory) and abiotic environmental factors that had driven the adaptive changes (Agrawal and Fishbein 2006). Identifying inter- and intraspecific variation in plant resistance is important to interpret chemical biological function, and to develop a better understanding of how plants interact with their herbivores. Not only does this have biological significance but it can also have applied relevance; for example, known chemical variation may be exploited in managed systems in an effort to reduce loss of tree productivity to herbivory, such as, the use of more resistant genotypes in commercial tree plantations (Miller *et al.* 2009), or for landscape restoration purposes. Despite extensive research on plant-herbivore interactions, there are gaps in the mechanistic understanding of variation in plant defence. There is also much that is unknown about the variation and consequence of recovery mechanisms in response to defoliation by herbivores. The key points raised in this opening paragraph are now introduced in more detail to provide a background to the experimental research I present in this thesis. The chapter concludes with a description of the study system that I employed and my research aims.

1.2 Chemical defence against herbivores

The adaptive significance of plant secondary metabolites (PSMs) is that they are believed to be primarily associated with defence against herbivores (Rosenthal and Berenbaum 1991; but see Hagerman *et al.* 1998; Close and McArthur 2002). They may be either be constitutive, always present in the plant; or induced, produced or translocated following damage or stress (Karban and Thaler 1999). Plant secondary metabolites have been grouped according to their biosynthetic origins; nitrogen containing compounds, phenolics and terpenoids (Harborne 1991). Alkoloids are the most well-known nitrogen-containing compounds; they have toxic effects on

their consumer that include inhibition of mitosis and DNA/RNA synthesis (Howe and Westley 1988). These metabolites are heterocyclic molecules that contain nitrogen (Harborne 1991). Phenolics are aromatic structures with one or more hydroxyl groups (Harborne 1991), the best known being tannins which can reduce herbivore digestibility of food by interfering with nitrogen availability by complexing with proteins, enzymes and carbohydrates (Foley *et al.* 1999; Iason 2005). Terpenoids are mainly cyclic unsaturated hydrocarbons, oxidised with substituent groups attached to the base of the carbon skeleton (Harborne 1991). Terpenoids are a large and diverse group of PSMs. They have been shown to act directly as a toxic load when consumed (McLean and Foley 1997), alter feeding behaviour (Wiggins *et al.* 2003), act as a volatile cue to deter herbivores (Lawler *et al.* 1999b; Bedoya-Pérez *et al.* 2014) or may act indirectly by attracting herbivore predators to protect the plant (Kessler and Heil 2011). There are major groups of terpenes, including mono- and sesquiterpenes, which are determined by the number of carbon isoprene units in their structure. These compounds are synthesised through two distinct biochemical pathways. The mevalonic acid pathway, located in the cytosol, produces the precursor of sesquiterpenes (C15), while the deoxyxylulose phosphate/methylerythritol pathway, in the plastids, produces the precursor of monoterpenes (C10). Further modification of these precursors occurs through secondary processes, such as oxidation, to produce a diverse range of plant terpenes (Huber and Bohlmann 2004; Degenhardt *et al.* 2009).

1.3 Variation in chemical defence

Chemical defence may be driven by genetics, plant development and may change in response to abiotic variables (Berenbaum and Zangerl 1992; O'Reilly-Wapstra *et al.* 2005a; Ballhorn *et al.* 2011; Gutbrodt *et al.* 2012; Holeski *et al.* 2012; Kariñho-Betancourt *et al.* 2015) . In this thesis I focus on quantitative genetics and plant development. Topical research on plant-herbivore interactions aims to determine the relevance of such variation, and to understand its community wide effects. For example, genetic-based differences in foliar chemistry may alter associated assemblages of consumers (Whitham *et al.* 2006; Barbour *et al.* 2009c). Genotypic variation in foliar chemistry has been widely documented in natural populations

(Berenbaum *et al.* 1986; Marquis 1990; Singer and Parmesan 1993; Hwang and Lindroth 1997; Hjalten *et al.* 2000; O'Reilly-Wapstra *et al.* 2004; Külheim *et al.* 2015). That variation may be interpreted in an eco-evolutionary context in which herbivores can select for defence traits in plant populations where genetic variation for these traits exists (e.g. Shelton 2004; Moore *et al.* 2014). Genetic variation in chemical defence is mediated by a range of biotic and abiotic selection pressures acting over different spatial and temporal scales (Futuyma and Agrawal 2009). Such variation may occur between evolutionary lineages of a species, between species within a lineage (Agrawal 2007) and also at multiple scales within a species, e.g. between different populations and families within populations (Armbruster 1991; O'Reilly-Wapstra *et al.* 2013b; O'Reilly-Wapstra *et al.* 2014a). In this scenario, selection pressures that vary even under small scales may generate strong local divergences in the expression of certain phenotypic traits.

The second driver of variation in chemical defence addressed in this thesis is plant development. Since plants experience different resource and physical constraints during their life time, it is also expected that variation in defence strategies will differ with plant age or ontogeny (Boege and Marquis 2005). Understanding such variation will help identify selective pressures posed by herbivores as plants develop. For example, an inconsistent expression of chemical defence across different life stages may be a response to multiple herbivores (Iason *et al.* 2011). In contrast, a consistent pattern in defence may arise if plants employ a similar suite of defences to protect themselves against multiple herbivores or if they suffer attack from a single herbivore across multiple stages (Leimu and Koricheva 2006). Varying ontogenetic trajectories in defensive chemistry has also been shown to alter interactions between the plant and higher trophic levels (Quintero *et al.* 2014).

1.4 Plant recovery

In the event of herbivore attack, many plants employ recovery mechanisms to reduce the negative effects of defoliation. Recovery through vegetative growth in woody plants is sourced from buds and meristems and may be initiated in response to a number of disturbance types including herbivory, fire and draught (Noble 2001; Burrows 2013; Clarke *et al.* 2013; Nzunda *et al.* 2014; Pausas and Keeley

2014; Shibata *et al.* 2014). Such responses may be facilitated by a range of physiological mechanisms such as increasing leaf photosynthetic rates in residual plant tissue and altered allocation of photoassimilate across plant organs (Stevens *et al.* 2008). When assessing the fitness effects of such responses to defoliation, this is known as plant tolerance (Strauss and Agrawal 1999; Simms 2000; Tiffin 2000), and together with resistance is considered to be a key aspect of plant adaptation to herbivory (Stowe *et al.* 2000; Fornoni 2011). Identifying traits of tolerance is challenging because it is defined operationally as the association between fitness and damage in a group of genetically related plants, such as resource allocation patterns, plant architecture and photosynthetic activity (Trumble *et al.* 1993; Strauss and Agrawal 1999; Haukioja and Koricheva 2000; Stowe *et al.* 2000; Tiffin 2000; Fornoni *et al.* 2003; Stevens *et al.* 2008). To fit this definition, expressions of tolerance have been quantified by comparing traits before and after damage. Studies involving these traits can help identify allocation and ecological constraints that limit the extent to which tolerance can respond to selective forces, but to do so, there is a need for empirical studies on plants with different genetic backgrounds (Fornoni 2011; Shibata *et al.* 2014). In addition, we need to assess the consequence of recovery mechanisms on plant phenology and how they may alter the associated biotic community, i.e. indirect effects (Ohgushi 2005; Hrabar and Du Toit 2014; Stam *et al.* 2014).

1.5 Seedlings: consequences of herbivory

Plants are often most vulnerable to herbivory in their early stages of growth (Crawley 1989; Lauda *et al.* 1990), and damage during this time will reduce plant fitness through increased mortality, reduced reproductive age and lifetime reproductive success, or declined competitive ability (Prins and Nell 1990; Stearns 1992; Bulmer 1994; Roughgarden 1998). Such changes can have dramatic flow-on consequences to the associated biotic community, such as altering the distribution and abundance of plants (Huntly 1991; Bryant *et al.* 1992; Barton and Hanley 2013). Herbivores are likely to remove a relatively greater amount of a juvenile plant's biomass and growing points than from a larger, reproductively mature conspecific. The relative costs of damage are therefore magnified, and the

deployment of defence strategies at the early establishment stage is likely to be selected for (Stowe *et al.* 2000). Indeed, many studies have demonstrated the juvenile stage to be the better defended (Reichardt *et al.* 1984; Tahvanainen *et al.* 1985; Glen *et al.* 1990; Morse *et al.* 1991; Wallace and Eigenbrode 2002; Loney *et al.* 2006). However, the pattern of ontogenetic change in PSM content that occurs when plants grow from juvenile to adult stages is inconsistent between species (Boege and Marquis 2005) with some evidence of greater resistance in adult stages (Bryant and Julkunen-Tiitto 1995; Dominy *et al.* 2003; Goodger *et al.* 2004; Boege 2005a; Kariñho-Betancourt *et al.* 2015). Plant response to herbivore attack may also vary during plant development, and provides an important source of variation in recovery traits (Boege 2005b; Barton and Hanley 2013; Massad 2013). Such ontogenetic shifts may be driven by plant resource allocation and acquisition priorities, such that early growth stages have higher leaf:stem and leaf:root mass ratios than later stages, and therefore a limited storage capacity with which to facilitate recovery. Despite this, and their small size, seedling and juvenile stages of many plant species exhibit a remarkable ability to survive after suffering even severe loss of biomass (Noble 1984; Hikosaka *et al.* 2005; da Silva Alabarce and Dillenburg 2014; Nzunda *et al.* 2014; Aparicio *et al.* 2015). To understand the adaptive potential for increased recovery in young plants, there is a need for genetic-based morphological data to shed light on the constraints and mechanisms of recovery.

1.6 The *Eucalyptus* system

Eucalypts (genus *Eucalyptus*) are dominant trees in most Australian forest types (Williams & Brooker 1997) and a globally important plantation tree (Myburg *et al.* 2014). The quantitative genetics and ecology of eucalypts have been well studied, allowing fundamental questions to be answered relating to plant-herbivore interactions (Lawler *et al.* 2000; Moore and Foley 2005; Wiggins *et al.* 2006a; Miller *et al.* 2007; Barbour *et al.* 2009c; Andrew *et al.* 2010; O'Reilly-Wapstra and Cowan 2010; O'Reilly-Wapstra *et al.* 2014a). During their lifetime, eucalypts are confronted with a multitude of biotic and abiotic stresses. They are susceptible to damage by fungal pathogens (Keane *et al.* 2000), and a range of invertebrate

(Jordan *et al.* 2002; Rapley *et al.* 2004a, c) and vertebrate herbivores (Bulinski 1999, 2000; Dungey and Potts 2002; Borzak *et al.* 2015a, chapter 5). These interactions have fitness consequences for eucalypts, such as increased mortality, growth and reproduction (Wilkinson and Neilsen 1995; Bulinski and McArthur 1999; Close *et al.* 2010; Eyles *et al.* 2013). These effects are often most pronounced following herbivore damage on early growth stage plants. In an applied context, studies have shown that mammal browsing on establishing plants leads to a decline in plantation productivity (Coleman *et al.* 1997) through reduced seedling growth rate and survival (Wilkinson and Neilsen 1995), and by changing tree form (Bulinski and McArthur 1999; Close *et al.* 2010). Early growth has also been significantly correlated with the probability of later age survival (i.e. size dependent mortality; Chambers *et al.* 1996) and growth (e.g. Stackpole *et al.* 2010). Since *Eucalyptus* is globally important in commercial plantations and landscape restoration, understanding its defence syndrome during early growth stages is valuable for the development of management strategies to reduce loss of plant fitness to herbivores during establishment.

Eucalypt foliage contains a diverse range of PSMs, including terpenes, phenolic compounds (e.g. condensed tannins) and formylated phloroglucinol compounds (FPCs; e.g. sideroxylonal A; Brophy and Southwell 2002; Moore *et al.* 2004). Many of these compounds have been shown to be important in reducing browsing by mammal herbivores (Lawler *et al.* 1998; Lawler *et al.* 2000; Wallis *et al.* 2002; Wiggins *et al.* 2003; McLean *et al.* 2004; O'Reilly-Wapstra *et al.* 2004; Burchfield *et al.* 2005; O'Reilly-Wapstra *et al.* 2005a). Whilst previous work on eucalypt defence has demonstrated genetic diversity in chemical composition (O'Reilly-Wapstra *et al.* 2004; O'Reilly-Wapstra *et al.* 2005b; Andrew *et al.* 2007b; Wallis *et al.* 2011; O'Reilly-Wapstra *et al.* 2013b; Padovan *et al.* 2014; Külheim *et al.* 2015), little is known of the genetic basis of ontogenetic development during the crucial early developmental seedling stage. Investigating such variation is important to identify the potential of ontogenetic changes in chemical resistance as a means of adaptation.

Following herbivore attack, eucalypts demonstrate a remarkable ability to recover through vegetative regeneration (Pinkard *et al.* 2006b; Eyles *et al.* 2009). Research in this system has demonstrated large effects of defoliation on biomass production and allocation in the short-term, however, in the longer term these often show little change as they recover through vegetative regrowth (Quentin *et al.* 2011b; Barry *et al.* 2012). Eucalypts have a number of sprouting structures including the axillary (in the axil of leaves), epicormic (in the older branches and stems) and lignotuber (in a woody swelling at the base of the stem) structures and will utilise these in that order with increasing intensity of damage (Burrows 2013). The type of storage utilised in recovery of woody plants is important in determining the mode of resprouting (Lawes and Clarke 2011), and will have substantial consequences for plant function and phenology. To support the costs of respiration and regrowth, eucalypts initiate physiological responses such as increased photosynthetic rates in residual leaves (Barry and Pinkard 2013; Lisboa *et al.* 2014). Previous studies have broadened our understanding of those mechanisms in facilitating recovery in eucalypts (Pinkard and Beadle 1998; Pinkard 2003; Eyles *et al.* 2009), and this work has helped to identify damage thresholds and management strategies to reduce the negative impact of herbivore damage in commercial plantations. Despite this, few studies have investigated the genetic basis of variation in eucalypt recovery responses (Whittock *et al.* 2003). This is important if we are to build on our understanding of fundamental ecological and evolutionary interactions between eucalypts and their herbivores. Such quantitative studies will add a further component to the damage thresholds and applied management strategies identified by current literature on eucalypt physiological responses to defoliation (reviewed by Eyles *et al.* 2013).

Mammal browsing clearly has the potential to change plant phenotype through the physiological mechanisms described above, however the growth and community consequences of such changes have received little attention. Eucalypt recovery from sprouting structures often leads to phenotypic changes (e.g. plant architecture Bulinski and McArthur 1999; Close *et al.* 2010) that may alter the associated biotic community (Steinbauer *et al.* 1998). To date, there is little research investigating the indirect impact of mammalian herbivores within eucalypt systems, and such

work is crucial when addressing the importance of mammals as ecological drivers in this system.

1.7 Study species

Eucalyptus globulus offers an ideal study system to investigate resistance and recovery as defence strategies against herbivores. It is a dominant tree in native forests in south-eastern Australia, and is found naturally in relatively moist coastal sites & well-drained frost-free valleys to 500m, and ranges from medium-sized trees in woodlands (15-20m high) to tall trees in open forests (up to 60m high (Cameron 1994). It is a major plantation species in temperate regions of the world (Dutkowski and Potts 1999) due to its many favourable characteristics, especially fast growth rate, high pulp yield and hard, durable timber. *Eucalyptus globulus* has received considerable research attention in relation to quantitative and molecular genetic variation in key plant traits (Potts *et al.* 2004). This allows genetic information to be linked with ecological studies to investigate selection by herbivores in a complex ecological and evolutionary framework (O'Reilly-Wapstra *et al.* 2014a). Molecular studies have shown that *E. globulus* comprises three major lineages - a mainland Australian lineage, an eastern-Tasmanian lineage and a western-Tasmanian lineage (Jones *et al.* 2013). Within these lineages, this species has been classified into 13 genetically-differentiated broad geographical groupings called races, and populations within these races are defined as trees growing within 10km of one another (Fig. 1.1; Dutkowski and Potts 1999). Families within populations are offspring from a single mother tree, which in the case of natural population samples is derived from open pollination (Potts and Jordan 1994). The experimental trials were grown using common garden designs, and whilst they were grown outside the climatic range of some of the *E. globulus* populations, the common environment ensures that any differences in assessed traits are due to underlying genetic effects.

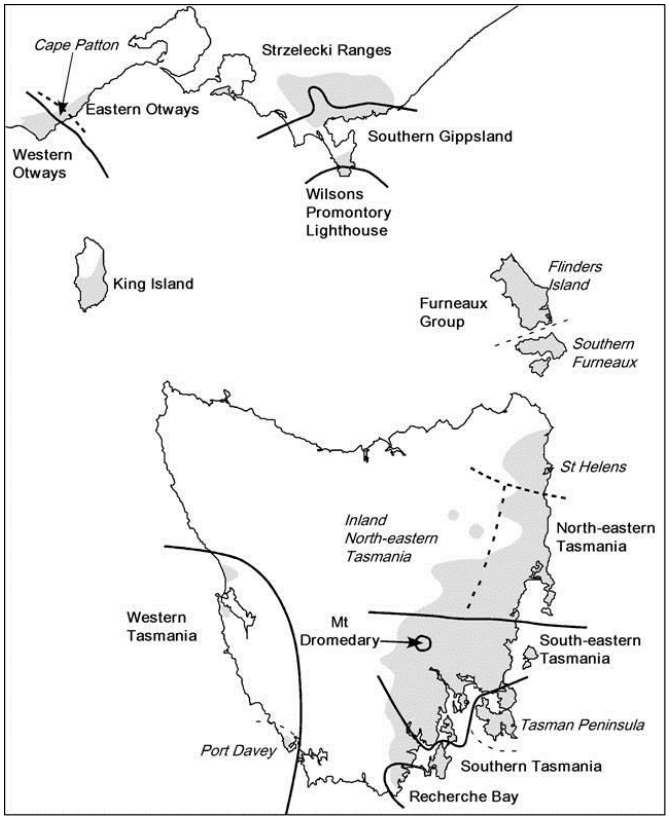

Figure 1.1. Native distribution of *E. globulus* populations in south eastern Australia (Dutkowski and Potts 1999). Races are separated by solid lines, and sub-races are shown in italics, separated by dotted lines.

Two important herbivores of *E. globulus* are the common brushtail possum (*Trichosurus vulpecula* Kerr, 1792) and the red-bellied pademelon (*Thylogale billardierii* Desmarest 1822) (Gilbert 1961). Brushtail possums do not have highly specific dietary preferences (Freeland and Winter 1976; Fitzgerald 1984), although some studies have shown that they exhibit strong preference for eucalypt foliage (Statham 1983; Fitzgerald 1984; McArthur and Turner 1997; Procter 1998), with a higher consumption of juvenile compared to adult foliage (Loney *et al.* 2006). The red-bellied pademelon are relatively generalist herbivores with a low preference for eucalypt foliage (Statham 1983; Jarman and Phillips 1989). However, some preference has been observed in *E. nitens* feeding trials, where pademelons preferred older seedling leaves with lower concentrations of sideroxylonals and cineoles (Loney 2007). Both the brushtail possum and red-bellied pademelon cause widespread and significant browsing damage in Tasmanian eucalypt plantations (Coleman *et al.* 1997; Bulinski 1999). Mammalian herbivores cause economic

damage in newly established plantations by eating leaves and stems of seedlings (Cremer 1969; Statham 1983; Wilkinson and Neilsen 1995; Montague 1996; Coleman *et al.* 1997; Bulinski and McArthur 1999). Chemical defence against herbivory and recovery mechanisms following damage are likely to be strongly selected at the vulnerable seedling and sapling stage, and this early developmental stage is the focus of this thesis.

1.8 Overview of chapters

The aim of this thesis is to strengthen our understanding of intraspecific and within-plant variation in chemical defence of early growth phases in *E. globulus*, and to investigate the importance of recovery mechanisms to counter the negative effects of herbivory. To achieve this I assess the genetic-based variation in foliar PSMs during early plant development, and investigate the damage and consequence of both natural and simulated defoliation damage, as detailed in the chapter outlines below. Experiments in chapters 2-4 use plants from the same native populations and families, to determine the pattern of variation in plant chemistry, physiology and morphology in response to different browsing treatments, and provide insights into the differences between these populations. Chapter 5 uses common environment field trial using controlled pollinated derived full-sib families (known as mother and father) that were originally established for applied breeding purposes, and provides control for genetic and environmental variation. Exploring interactions between mammals and eucalypts will greatly add to our understanding of plant responses and their consequences for trophic interactions and ecological communities in a broader ecophysiological framework. This study is also significant from a management perspective. Improved understanding of critical damage levels and recovery responses contribute to efficient and ethical management strategies to reduce herbivore damage in commercial plantations and restoration projects.

Specifically I ask the following questions:

1. Is there ontogenetic variation in foliar terpenes during early stage development of *E. globulus* seedlings, and does this vary at the population and family level?
2. Is there intraspecific genetic variation in photosynthetic rate, chlorophyll content, defensive chemistry and growth response to partial artificial browsing of juvenile *E. globulus*.
3. Is there intraspecific genetic variation in *E. globulus* seedling survival and growth from lignotubers after severe artificial browsing.
4. What is the pattern (spatial and genetic) and direct impact of natural mammalian browsing on *E. globulus* seedlings in the field, and subsequent indirect effects on a dependent foliar organism.

Chapter 2 determines the genetic-based ontogenetic variation in foliar terpene composition of early stage *E. globulus* seedlings. Terpenes are important chemical components of defence against eucalypt herbivory and this chapter explores population differences in ontogenetic change of terpenes in *E. globulus*. In a glasshouse trial, I use three populations that express inherently different levels of terpenes, and assess content in leaves harvested at the cotyledon-stage, and consecutive pairs of true leaves across an eight month period. This sampling methodology separates the effect of ontogeny from leaf physiological aging, which are two important processes not often addressed in studies of foliar chemical defence.

Chapter 3 explores the genetic variation in the physiological responses to a 50% defoliation treatment of *E. globulus* seedlings. Increased photosynthetic rate and chlorophyll content are commonly employed mechanisms of eucalypts to aid recovery from foliage loss, but to date the genetic control of such responses is not known and there is little knowledge of the chemical profile of eucalypt regrowth from axillary buds following damage. Investigating such responses to defoliation in a genetic context is important to improve our understanding of the selective pressures of herbivores. In a nursery environment, I use the same three *E. globulus* populations as in Chapter 2 to investigate the genetic-based physiological response

in the new and remaining foliage 12 weeks after defoliation treatment, including foliar chemical content.

Chapter 4 investigates the intraspecific variation in two *E. globulus* populations in seedling recovery from lignotubers after severe artificial browsing. Intraspecific variation exists in both lignotuber development and the expression of chemical resistance in this species, but it is not known if plant recovery differs between these two populations. In this chapter I investigate the genetic difference in growth/recovery from severe defoliation in the short term (nursery trial) and long-term (field trial grown to 32 months after treatment), and discuss the results according to resource allocation theory.

Chapter 5 describes the pattern (spatial and genetic) and direct impact of uncontrolled mammalian browsing on *E. globulus* seedlings, and assesses the subsequent indirect effects on a dependent foliar organism. In a large field trial I assess the genetic basis of herbivore preferences, the impact of single and repeated browsing on tree fitness and morphological traits, and the associated indirect plant-mediated effects on a subsequent herbivore, the autumn gum moth. This chapter highlights the importance of incorporating whole communities in plant-herbivore studies as the direct effects of browsing have the potential to influence not only the biotic community structure of a foundation species host-plant, but also the evolutionary interactions that occur between organisms and the host-plants themselves.

Chapter 6 is a synthesis of the four key experimental results: 1). clear responses that occurred in multiple components to defoliation of *E. globulus* seedlings; 2). genetic stability in many chemical and plant recovery responses amongst divergent populations of *E. globulus*; 3). while many of the response patterns were stable amongst populations, there were several notable exceptions involving ecologically important traits. 4). many of the chemical responses observed could be linked to their biosynthetic origins. Finally, these results are discussed as a collective, to summarise how they have advanced our knowledge in the field as well as potential future directions.

Chapter 2:

Population divergence in the ontogenetic trajectories of foliar terpenes of a *Eucalyptus* species

This chapter has been published as:

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2.1 Abstract

The development of plant secondary metabolites during early life stages can have significant ecological and evolutionary implications for plant-herbivore interactions. Foliar terpenes influence a broad range of ecological interactions, including plant defence, and its expression may be influenced by ontogenetic and genetic factors. This study investigates the role of these factors in the expression of foliar terpene compounds in *Eucalyptus globulus* seedlings.

Seedlings were sourced from ten families each from three genetically distinct populations, representing relatively high and low chemical resistance to mammalian herbivory. Cotyledon-stage seedlings and consecutive leaf pairs of true leaves were harvested separately across an eight month period, and analysed for eight monoterpene compounds and six sesquiterpene compounds.

Foliar terpenes showed a series of dynamic changes with ontogenetic trajectories differing between populations, families, as well as between and within the two major terpene classes. Sesquiterpenes changed rapidly through ontogeny, expressed opposing trajectories between compounds but showed consistency in pattern between populations. Conversely, changing expression in monoterpene trajectories were population and compound specific.

The evidence presented here suggests that there exists adaptive opportunities for changing levels of terpene content through ontogeny, and evolution may exploit the ontogenetic patterns of change in these compounds to create a diverse ontogenetic chemical mosaic with which to defend the plant. We hypothesise that the observed genetic-based patterns in terpene ontogenetic trajectories reflect multiple changes in the regulation of genes throughout different terpene biosynthetic pathways.

2.2 Introduction

Ontogenetic development in plants results in changes in the expression of key plant traits (Barton and Koricheva 2010) such as plant secondary metabolites (PSMs; Barton 2007; Holeski *et al.* 2012; Barton and Hanley 2013). Many PSMs play important roles in the defence of plants against natural enemies and understanding how PSMs and resistance vary across ontogenetic stages offers insight into the selective impacts of herbivores and pathogens on plant traits such as 'who' is acting as the selective force, 'when' is selection occurring and 'what' is the result of selection on the plant traits (e.g. directional or stabilising selection; Boege and Marquis 2005).

Patterns in chemical expression across ontogenetic stages appear variable. For example, as some plants age from seedling to juvenile through to adult stages there is an increase in the expression of PSMs (e.g. Elger *et al.* 2009) whilst others decrease with plant age (e.g. Goodger *et al.* 2006). Such variability in ontogenetic trajectories may be influenced by plant life-history strategy, investment in defence trait type (chemical, physical and/or tolerance), the type of herbivore or pathogen acting as the selective agent and the timing of enemy attack (Barton and Koricheva

2010; Massad 2013; Quintero and Bowers 2013). The mechanisms behind the different ontogenetic patterns have been explained by two hypotheses. The herbivore selection theory predicts a high level of defence in juvenile plants, followed by a decrease as plants mature and become less susceptible to the reduction in fitness caused by these attacks (Bryant *et al.* 1992). A contrasting prediction based on the allocation theory suggests that the acquisition and allocation of resources limit the production of defensive secondary compounds in young plants, thereby resulting in an ontogenetic increase in defence (Herms and Mattson 1992).

Terpenes are a large and diverse group of plant secondary metabolites, and can have pronounced effects on a wide range of ecological interactions. For example, terpenes can influence plant-animal interactions by influencing insect community composition (Xiao *et al.* 2012), acting as attractants for specific pollinators (Ibanez *et al.* 2010; Reisenman *et al.* 2010; Piechowski *et al.* 2011), or in plant defence (Lawler *et al.* 2000; Wiggins *et al.* 2003; Ott *et al.* 2011). They can mediate plant-plant interactions by having belowground negative allelopathic effects (Singh *et al.* 2009; De Martino *et al.* 2010), affect ecosystem process through promoting fire (Ormeño *et al.* 2009; Zhao *et al.* 2011), and they can play important roles in microbial (Safaei-Ghomi and Batooli 2010; Huang *et al.* 2012) and fungal interactions (Ludley *et al.* 2009; Kramer and Abraham 2012). This is no better seen in the terpene rich trees of the southern hemisphere genus *Eucalyptus* (Steinbauer 2010; Myburg *et al.* 2014).

Eucalypts are the dominant tree species in many Australian ecosystems, ranging from dwarf mallee forms in high altitude and semi-arid regions through to tall forest trees in wet forests (Williams and Brooker 1997). Eucalypt foliage is high in concentration of PSMs including an array of mono- and sesquiterpene compounds (Boland *et al.* 1991; Brophy and Southwell 2002; Külheim *et al.* 2011). These compounds are known to influence a range of interactions with invertebrate (Edwards *et al.* 1990; Edwards *et al.* 1993; Steinbauer *et al.* 2004; Östrand *et al.* 2008; Steinbauer 2010) and vertebrate (Lawler *et al.* 1999a; Wiggins *et al.* 2003) herbivores. Terpene concentrations in eucalypts exhibit variation between and

within populations (O'Reilly-Wapstra *et al.* 2002; Wallis *et al.* 2002; Andrew *et al.* 2005; Wiggins *et al.* 2006b) and are highly heritable (Doran and Matheson 1994; Andrew *et al.* 2005; O'Reilly-Wapstra *et al.* 2005b; O'Reilly-Wapstra *et al.* 2011). Despite the ecological importance of eucalypts and the large number of terpene compounds in their foliage, few studies to date, have examined ontogenetic variation of terpene expression across distinct life stages (e.g. seedlings, juvenile, adult stages; O'Reilly-Wapstra *et al.* 2007; Goodger and Woodrow 2009) or within a single life stage (McArthur *et al.* 2010; Goodger *et al.* 2013a,b; Padovan *et al.* 2013). In addition, few studies in any system have examined the genetic basis to ontogenetic variation in PSMs (Barton 2007; O'Reilly-Wapstra *et al.* 2007; Holeski *et al.* 2009; Holeski *et al.* 2012). To the best of our knowledge, no studies have explicitly documented genetic variation in the ontogenetic expression of foliar terpenes within the early stages of seedling development (i.e. is there a genetic-based difference in the early ontogenetic development of chemical defence within the species). By placing ontogenetic patterns in a genetic framework, we better understand the evolutionary implications of the development of ecologically important PSMs. In this study, we report on variation in the ontogenetic trajectories of 14 different mono- and sesquiterpene compounds during early development of *Eucalyptus globulus* seedlings, and identify genetic differences in these trajectories at the population and family levels.

Eucalyptus globulus has been the subject of considerable research on the quantitative genetic control of important plant traits, including terpenes (O'Reilly-Wapstra *et al.* 2005a; Külheim *et al.* 2011; O'Reilly-Wapstra *et al.* 2011; Wallis *et al.* 2011). This species is heteroblastic and genetic variation has been detected both within and between populations at the juvenile coppice (O'Reilly-Wapstra *et al.* 2002) and adult (Wallis *et al.* 2011) leaf stages, and strong correlations between foliar concentrations across life history stages (juvenile and adult foliage types) have been reported at the population level (O'Reilly-Wapstra *et al.* 2007). To date, however, little is known of the pattern of expression of terpenes in the important early life stage of the seedling (McArthur *et al.* 2010; Goodger *et al.* 2013a), as is the genetic-based variation in ontogenetic development in terpenes. Focusing on the development of terpenes during the seedling phase is important as this is when

E. globulus, are often most vulnerable to herbivory and hence can have significant impacts on tree growth and fitness (Farrow *et al.* 1994; O'Reilly-Wapstra *et al.* 2012). Furthermore, few studies have investigated plant defence in the cotyledon stage, and this is important to determine how plant defence changes during the earliest ontogenetic stage (Barton and Koricheva 2010).

Three specific questions were addressed:

1. Is there ontogenetic variation in foliar terpene content of early stage seedlings?

2. Is there genetic-based variation at the population and family level in the ontogenetic trajectory of terpene concentration?

3. Do mono- and sesquiterpenes follow the same ontogenetic trajectory?

2.3 Materials and Methods

2.3.1 Trial design

The natural range of *Eucalyptus globulus* has been classified into 13 genetically differentiated geographical races (Dutkowski and Potts 1999). Populations within these races have been defined as trees growing within 10km of each other (Potts and Jordan 1994), and we define family as progeny derived from open-pollinated seed collected from one native tree within a population. We chose three populations from three different geographical races for this study, each represented by ten families. The chosen populations express inherently low and high concentrations of terpenes that confer resistance to mammal browsing (O'Reilly-Wapstra *et al.* 2004; O'Reilly-Wapstra *et al.* 2005a). The two Tasmanian populations chosen were St Helens in the northeast of Tasmania (41’ 15 S 148’ 19 E) with low PSM foliar chemistry and Blue Gum Hill in the southeast of Tasmania (43’ 03 S 146’ 52 E) with relatively high PSM foliar chemistry (O'Reilly-Wapstra *et al.* 2004). A third population, Jeeralang North in the Strzelecki Ranges of Victoria (38’ 19 S 146’ 52 E) also has higher levels of PSM foliar chemistry and low mammal browsing susceptibility (O'Reilly-Wapstra *et al.* 2004) and was chosen to represent a population that is from a distinct molecular group, to encompass the broad spatial genetic structure of this species (Jones *et al.* 2013). The trial was grown in a common environment glasshouse, well ventilated, no

additional lighting, with average temperatures ranging from 21˚C to 24˚C during the day, and 13.5˚C – 16.2˚C overnight. Seeds were sown in family lots in October 2009, in polystyrene boxes on soil covered in 0.5cm vermiculite. Standard low phosphorus potting mix was used, containing slow release fertiliser (N:P:K 17+1.6+8.7). These family trays were randomised in the glasshouse and plants grown for 28 days until cotyledons were fully expanded. Cotyledon-stage seedlings were pricked out into separate cells in 8x7 cell plastic seed trays. Fourteen cotyledon-stage seedlings per family were pricked out into two consecutive rows in these trays and these two rows comprised a 'family plot'. Ten such plots of each of the thirty families were pricked out and arranged into ten replicate blocks within the glasshouse. Each family was thus represented as a family plot of fourteen plants, with the plot arranged in a random position within each replicate block (3 populations x 10 families x 14 seedlings x 10 replicates = 4200 seedlings in total). Over the course of the experiment, replicates were moved three times to different positions in the glasshouse. The remaining cotyledon-stage seedlings (not used in the replicated design) were harvested to investigate their chemical content. Approximately sixty cotyledon pairs, pooled within family (25 families; insufficient sample size for 5 families) were harvested, discarding the stem, and placed into a freezer in labelled plastic bags for later chemical analysis.

2.3.2 Leaf harvest

It is important to separate the effect of ontogeny from leaf physiological ageing (Lawrence *et al.* 2003), as they are two different processes operating within the plant, and are often not separated (Diggle 2002; Goodger *et al.* 2013b). Leaf age was accounted for by sampling seedlings at multiple times through their growth, by separating consecutive leaf pairs and then comparing the same aged leaves from different nodes across different harvests of different seedlings within a family plot sampled at different times. Leaves at each node are opposite at this seedling stage and were grouped into pairs (2 leaves) to represent a specific ontogenetic and physiological aging stage (Fig. 2.1). Seasonal variation (e.g. in photoperiod) is also known to influence terpene content within the Myrtaceae (Simmons and Parsons 1987; Leach and Whiffin 1989; Wildy *et al.* 2000), but although seasonal change

could not be fully controlled in this experiment, it is expected to be relatively small and would be confounded in the leaf age by ontogeny interaction term.

Harvesting of seedling leaves began 20 days after sowing and continued for a period of 4 months as each leaf pair became fully expanded (48, 54, 62, 82, 98, 113 days). A final harvest was collected at 8 months (242 days; Fig. 2.1). As is typical in eucalypt literature (e.g. Brooker and Kleinig 1999; Goodger *et al.* 2013a), we define seedlings as the combined cotyledon stage and growth up to five nodes. All leaves were morphologically characteristic of the seedling growth stage of *E. globulus*, and harvested before the onset of the relatively stable juvenile leaf stage (Jordan *et al.* 2000). Seedlings were destructively harvested to avoid the possibility of induction affecting subsequent terpene expression. At each harvest, one of the fourteen seedlings in each family plot was selected for harvest, with the exception of the first two harvests where the leaves from four seedlings were combined due to their small size. Seedling leaves were cut from the stem using fine tip scissors, placed into labelled bags and kept in a cool box with ice until being transferred to a freezer for later chemical analysis. The bottom leaf pairs on older plants senesced naturally, and so to maintain a balanced statistical design only the top three leaf pairs from each plant were analysed. They were identified as young, intermediate or old in age (Fig. 2.1).

2.3.3 Foliar terpene analysis

At each harvest time, leaves for chemical analysis were pooled by leaf pair and pooled for each family across replicates to increase the sample quantity (i.e. 3 populations x 10 families = 30 bags of leaves for each leaf pair). Environmental variation within the glasshouse was minimal for the duration of the experiment and therefore pooling each family across replicates was the preferred method to boost sample size, as it still allowed for testing genetic variation at both the population and family level. Eight monoterpenes (1,8-cineole, α-pinene, limonene, α-terpineol, α-terpinyl acetate , *p*-cymene, terpinene-4-ol, 2-hydroxy-1,8-cineole) and six sesquiterpenes (aromadendrene, bicyclogermacrene, alloaromadendrene, α-gurjunene, β-caryophyllene, α-humulene) were quantified. Terpenes were extracted from leaves following a method modified from O'Reilly-Wapstra *et al.* (2004).

Results for 1,8-cineole and α-pinene were expressed in milligrams per gram of Dry Matter (mg g^{-1} DM) after calibration with reference standards.

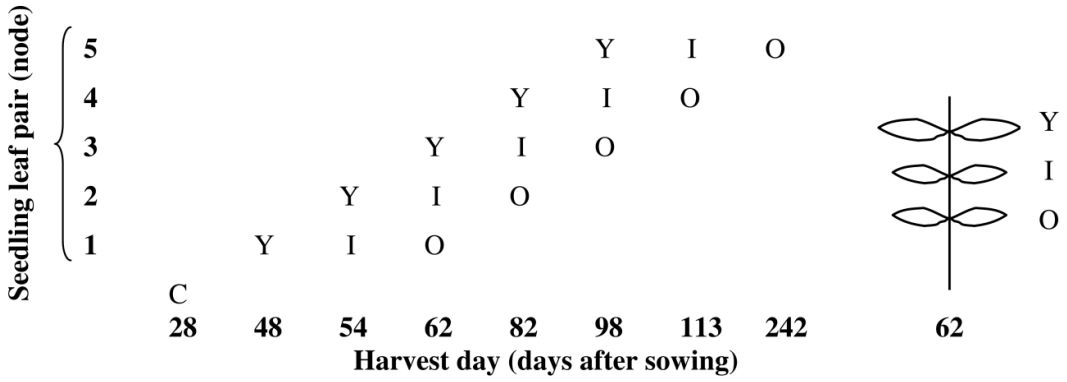

Figure 2.1. Experimental design of the trial indicating the time of leaf harvest, beginning with cotyledon-stage seedlings (C). The first five consecutive true leaf pairs are the ontogenetic stages examined. Leaf pairs were identified as either young (Y), intermediate (I) or old (O) in age to represent the physiological aging of each leaf. The illustration on the right is an example of the harvested seedling at 62 days with three nodes and three leaf pairs. Each leaf pair per harvest was represented by thirty *E. globulus* families from three populations (total n samples =450).

Total terpenes and the remaining compounds were expressed as mg g^{-1} DM cineole equivalents. Leaves were cut into small pieces (approx. 3-5mm^2) using scissors and ~0.25 g weighed in a 20 mL polypropylene tube. Due to the low terpene content of cotyledon-stage seedlings and first true leaves, the fresh leaf sample was doubled to ~0.5 g. Entire leaves (including mid-rib) were used for the first three harvests, after which the leaf midrib was not included. Fifteen mL of dichloromethane (DCM) spiked with an internal standard of n-heptadecane ($C_{17}H_{36}$) was added to each tube and left for one hour. The tubes were then placed in an ultra-sonic bath to sonicate for 30 minutes. The extract was decanted into a 50 mL tube and capped. This process was repeated another two times, pooling extracts. The pooled extracts were then mixed thoroughly. A pipette was used to take ~1 mL from each sample and placed in HPLC vials and refrigerated until analysis. The extracts were then analysed with gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC analyses were carried out on a Varian-450 GC with

Varian 1177 split/splitless injector and Flame Ionization Detector (FID). The column was a Varian 'Factor Four' VF-5ms (30 m x 0.25 mm internal diameter and 0.25 μm film). Carrier gas was nitrogen at 1 mL/min constant flow mode. Injections of 1 μL were made using a Varian CP-8400 autosampler and a Varian 1177 split/splitless injector with a 15:1 split ratio. The injector temperature was 220ºC, and the FID temperature was 300ºC. The column oven was held at 60ºC for 1 minute, then ramped to 220ºC at 6 degrees per minute, then to 300ºC at 25ºC per minute with a 5 minute hold at the final temperature. Data was processed with Varian Galaxie software. Preliminary GC-MS analyses on several representative samples were carried out on a Varian 3800 GC coupled to Varian 1200 triple quadrupole mass spectrometer in single quadrupole mode. The GC conditions were as above except the carrier gas was helium. The ion source was held at 230ºC, and the transfer line at 310ºC. The range from *m/z* 35 to 350 was scanned 4 times per second. Data was processed with Varian Chemstation software. Initial compound identifications were made by comparison of both Kovats indices and mass spectra with commercial databases (NIST MS database) and an in-house specialist terpene MS database. The GC processing method was then constructed once the target peaks for integration had been selected. Individual peak areas were then assigned automatically by the Galaxie software, but all assigned peaks were visually inspected to ensure accurate peak integrations and identifications had occurred. Peak area ratios to the internal standard peak were determined, as was the percentage contribution of each targeted peak to the total terpene profile.

Prior to each harvest, plant height (taken from the cotyledon node to the base of the apical leaf), lamina length and leaf shape (length to width ratio) was measured to estimate of plant growth. Lamina length and leaf shape were assessed on the top leaf pairs of each seedling. One seedling per family per replicate was measured (n=300) one day prior to each harvest and is presented in Supplementary material in Appendices 1, Fig. S1. There was no growth or leaf morphology data for the final harvest at 242 days.

2.3.4 Statistical analysis

All terpene values were log transformed to meet assumptions of normality and homoscedasity. Total mono- and sesquiterpene content was calculated by the sum of the individual compounds. Terpene content of true leaves was analysed for the effects of ontogeny, leaf age and population and family nested within population genetic effects using mixed model procedure in SAS (PROC MIXED; SAS Institute Inc. 2002-2008). The fixed effects in the model were population, ontogeny, leaf age and all their interaction terms. *Family nested within population*, and its two-way interaction terms with *ontogeny* and *leaf age* were the random effects in the model. These random terms provided the error terms to test for population and associated interaction terms using Wald's F-test. To estimate the percentage variance explained by the fixed and random terms, all components were fitted into a randomised mixed model (PROC MIXED of SAS) and the variance components for each variable were estimated. Canonical discriminant analysis (PROC DISCRIM of SAS) was used to summarise the multivariate changes in leaf terpene data using the unique combinations of ontogeny (leaf node), age and population as the grouping factor. This analysis was based on nine individual terpene compounds (limonene, α-terpineol, α-humulene, α-gurjunene and bicyclogermacrene were removed due to their high intercorrelations with other compounds).

Variation in seedling height, lamina length and leaf shape was analysed by fitting a mixed model (PROC MIXED of SAS) with population, harvest day and their interaction as the fixed terms and *replicate*, *family within population* and the interaction term *harvest day by family within population* as the random terms. Variation in terpene content of cotyledon-stage seedlings was analysed by fitting a fixed effects model (PROC MIXED of SAS) with population as the fixed term with the random term representing the variation between family pools within populations. While not embedded in the seedling ontogeny experimental design, the families were grown randomly positioned in the same area of the glasshouse, allowing us to make comparisons between terpene content of cotyledon-stage seedlings and the first true leaf pair using a paired t-test.

2.4 Results

There were highly significant effects of ontogeny, genetics (at both population and family level), leaf age and their interactions on the majority of the terpene compounds in true leaves (Table 2.1 and Supplementary Table S1). Variance components show that population explained the greatest percentage of the variation for total terpenes, mono- and sesquiterpenes (Table 2.2). While ontogenetic effects were the next most important factor in explaining variance for total terpenes and sesquiterpenes, family within population was next most important for monoterpenes. Leaf age explained only a small percentage of the terpene variation (Table 2.2) and this is consistent with the canonical discriminant analysis where all three leaf ages effectively followed the same trajectory with an increase in terpene content through ontogeny (Fig. 2.2).

Table 2.1. Results of the mixed model analysis examining the genetic, ontogenetic and leaf age effects on terpene content in the first five consecutive true leaf pairs of *E. globulus* seedlings. Compounds are listed in order of dominance within terpene classes. Family within population and its interaction with ontogeny and leaf age were used as the error terms (see Supplementary material for significance of these random terms; Supplementary Table S1). Total mono- and sesquiterpene content was calculated by the sum of their individual compounds. Significance of effects indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001, and blank, not significant.

	Population $F_{2,27}$	Ontogeny $F_{4,108}$	Leaf age $F_{2,54}$	Population* Ontogeny $F_{8,108}$	Population* Leaf age $F_{4,54}$	Leaf age* Ontogeny $F_{8,210}$	Population*Leaf age*Ontogeny $F_{16,210}$
Total terpene	29.0 ***	101.0 ***	32.8 ***	10.6 ***	2.0	11.9 ***	3.0 ***
Total Monoterpenes	34.1 ***	47.0 ***	22.8 ***	13.2 ***	1.6	7.8 ***	2.5 **
Total Sesquiterpenes	44.6 ***	238.2 ***	32.6 ***	8.9 ***	0.4	8.6 ***	1.6
Monoterpenes							
1,8-cineole	27.3 ***	77.8 ***	23.0 ***	13.2 ***	1.4	7.1 ***	1.9 *
α-pinene	23.5 ***	30.7 ***	52.2 ***	7.1 ***	1.4	7.2 ***	2.4 **
limonene	33.7 ***	81.2 ***	94.4 ***	9.0 ***	0.7	13.7 ***	2.9 ***
α-terpineol	17.4 ***	49.9 ***	22.1 ***	15.7 ***	1.5	5.5 ***	1.4
α-terpinyl acetate	2.4	39.0 ***	1.5	17.3 ***	0.7	2.6 *	1.6
p-cymene	35.3 ***	14.5***	12.3***	52.7 ***	8.4 ***	13.7 ***	4.5 ***
terpinene-4-ol	33.4 ***	36.8 ***	21.5 ***	6.8 ***	2.0	7.8 ***	2.6 ***
2-hydroxy-1,8-cineole	10.8 ***	22.8 ***	67.9 ***	8.0***	8.2 ***	72.8 ***	0.9
Sesquiterpenes							
aromadendrene	53.7 ***	473.7 ***	10.8 ***	4.9 ***	1.2	1.5	1.2
bicyclogermacrene	50.2 ***	227.4 ***	49.1 ***	5.0 ***	1.2	31.4 ***	3.3 ***
alloaromadendrene	64.1 ***	382.2 ***	15.5 ***	2.8 **	0.7	0.9	0.6
α-gurjunene	55.4 ***	304.7 ***	65.5 ***	2.4 *	5.0 **	23.0 ***	2.4 **
β-caryophyllene	0.5	302.7 ***	74.8 ***	6.2 ***	0.8	4.7 ***	1.0
α-humulene	1.9	266.2 ***	97.8 ***	1.3	4.4 **	8.44 ***	2.9 ***

Table 2.2. Results of a fully random model showing the percentage contribution of genetic, ontogenetic and leaf age variance component estimates for total terpenes and the two major terpene classes in the first five consecutive true leaf pairs of *E. globulus* seedlings. Total mono- and sesquiterpene content was calculated by the sum of their individual compounds. Significance of effects indicated: **, $P < 0.01$; ***, $P < 0.001$, and blank, not significant.

Effect	Total Terpene	Monoterpenes	Sesquiterpenes
Population	35.0 ***	46.5 ***	39.8 ***
Ontogeny	22.0 ***	8.2 ***	35.8 ***
Leaf age	1.9 ***	1.4 ***	1.1 ***
Population*Ontogeny	6.6 ***	9.2 ***	3.7 ***
Population*Leaf age	0.0	0.0	0.0
Leaf age*Ontogeny	4.1 ***	2.5 ***	1.7 ***
Population*Leaf age*Ontogeny	2.4 ***	1.9 **	0.3
Family(Population)	11.5 ***	13.0 ***	8.3 ***
Family(Population)*Ontogeny	3.2 **	3.3 **	2.5 ***
Family(Population)*Leaf age	0.0	0.0	0.0
Residual	13. 3 ***	13.9 ***	6.8 ***

The canonical discriminant analysis based on 9 individual compounds (Fig. 2.2) showed a strong ontogenetic influence on terpene composition, with population being next most important. Canonical variate (CV) 1 explained the ontogenetic change, accounting for 61% of the variation ($F_{396,3475} = 11.1$, $P<0.001$) in terpenes between groups. CV 2 accounted for population differences as well as a component of the ontogenetic change in the Jeeralang population and explained 19% of variation ($F_{344,3105} = 7.1$, $P<0.001$). CV 3 (figure not shown) accounted for 10% of variation ($F_{294,2730} = 4.9$, $P<0.001$), although it mainly explains population variation and did not embody any ontogenetic change.

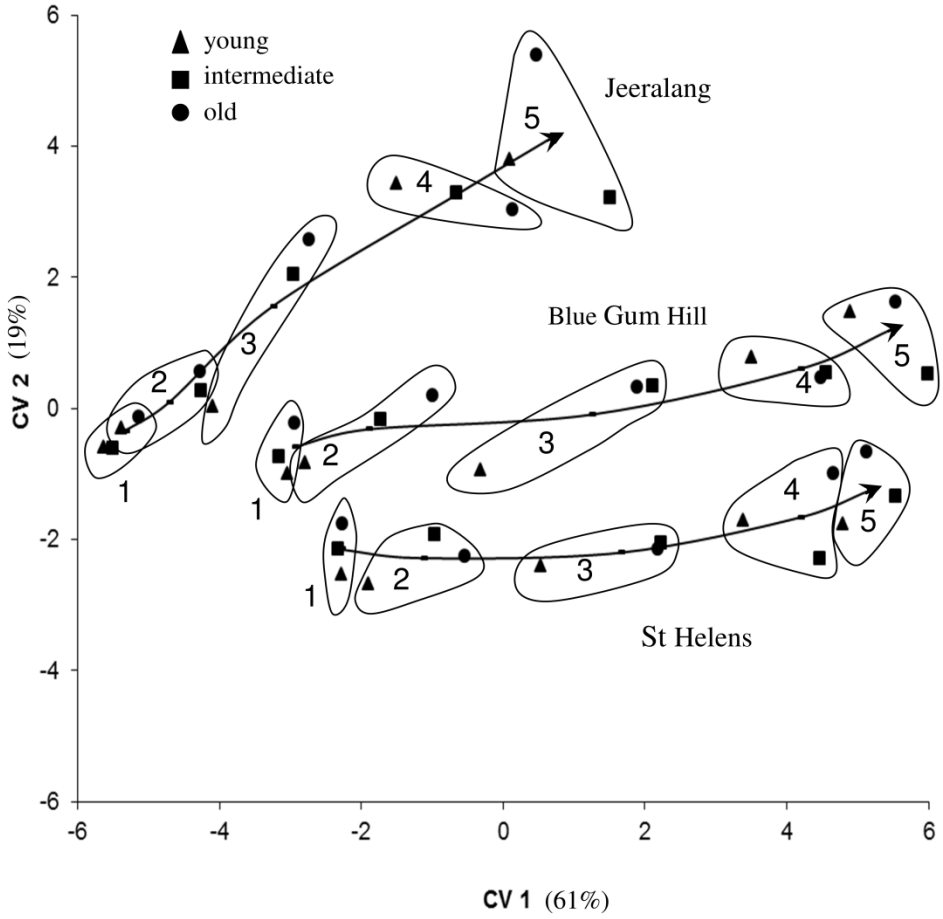

Figure 2.2. Results of the canonical discriminant analysis based on nine terpene compounds showing the chemical trajectory for each *E. globulus* population, and grouped by leaf age (triangles, circles, squares) and ontogenetic stage (1 to 5). Within each population, there are clear groupings of ontogeny (represented by the first five consecutive true leaf pairs), despite different leaf ages. The continuous lines for each population shows the average across leaf age for each ontogenetic stage (arrows indicate directionality). Cotyledon-stage seedlings were not included in this analysis.

These combined results indicate the importance of genetic and ontogenetic effects on these compounds in the early seedling stages of *E. globulus*. Leaf age showed significant two-way interactions with ontogeny, and three-way interaction with ontogeny and population (Table 2.1). The two-way interaction may be explained by a one node difference in the young leaves where they showed an ontogenetic change earlier (at node 3) than the older leaves (data not shown). The significant three-way interaction was due to the failure of the St Helens population to reach higher terpene

levels in later ontogeny (data not shown). The high effect (F-value) of leaf age by ontogeny interaction on 2-hydroxy-1,8-cineole content is explained by a rapid increase in the terpene content of the "old" leaf pair at the final harvest (day 242) in Jeeralang (data not shown).

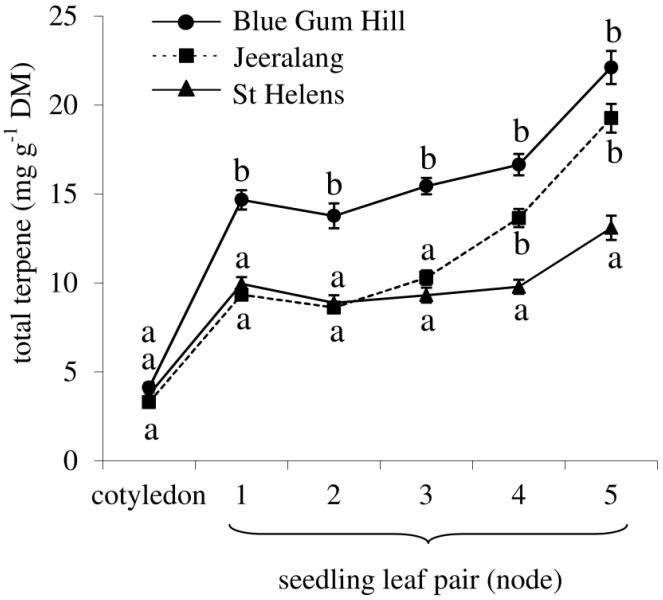

Figure 3. Population variation in the ontogenetic development of total foliar terpene in *E. globulus* seedlings from the cotyledon-stage (C) to node five. Ontogenetic stage is represented by cotyledons and the first five consecutive true leaf pairs. The figure shows different ontogenetic trajectories of the mainland Australian population, Jeeralang, compared to the two Tasmanian populations, St Helens and Blue Gum Hill. Letters that differ on each ontogenetic trajectory indicate values that are significantly different within population ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons).

The ontogenetic change in terpene content amongst the three *E. globulus* populations varied between terpene compounds. The two Tasmanian populations (Blue Gum Hill and St Helens) showed substantial differences in absolute total terpene and monoterpene content, but they shared a similar magnitude and direction of change through ontogeny (Fig. 2.2, 2 3 and 2.4). The mainland Australian population (Jeeralang) however, differed in the magnitude of change and the direction of trajectory through ontogeny (Fig. 2.2 and 2.3). This pattern is clear in the development of total terpenes where Jeeralang seedlings initially began their

development similar to St Helens but then rapidly increased to express levels more similar to Blue Gum Hill later in ontogeny (from leaf pair 4; Fig. 2.3). Total terpene content increased from the first to the fifth seedling leaf pair by 7.44 mg g^{-1} DM in Blue Gum Hill (1.5 fold increase); 3.13 mg g^{-1} DM in St Helens (1.3 fold increase), compared to a large 9.93 mg g^{-1} DM increase in Jeeralang (2.1 fold increase). The points of rapid change in leaf terpene content did not coincide with the continuous pattern of change in lamina length or leaf shape with node (Supplementary material in Appendices 1, Fig. S1 B and C). This suggests that the trends in leaf terpene content and leaf morphology throughout early development are independent.

There were significant population by ontogeny interactions for all terpenes (except α-humulene) in the true leaves, that showed consistent trends but with differences in the timing and rate of change. *p*-Cymene was the exception as one population (Jeeralang) differed in the direction of ontogenetic development to the others (Fig. 2.4A-H). This result was noted by the high population by ontogeny interaction effect in this compound (Table 2.1). The two Tasmanian populations remained stable in *p*-cymene content whereas Jeeralang increased after the second ontogenetic stage (Fig. 2.4F). In contrast, the monoterpenes 1,8-cineole, limonene and α-terpineol (Fig. 2.4 A,C,D) showed similar increases through ontogeny in all populations, but with differences in the timing and rate of change between populations. Decreasing levels were evident in α-terpinyl acetate with a sharply change in Jeeralang, relatively no change in Blue Gum Hill and an intermediate pattern in St Helens (Fig. 2.4E). The monoterpene α-pinene (Fig. 2.4B) also initially decreased in content in all populations, but then increased at different rates. The monoterpene terpinene-4-ol (Fig. 2.4G) showed a similar population ranking to *p*-cymene with a rapid increase in Jeeralang, whereas the Tasmanian population remained low and was relatively stable. The monoterpene 2-hydroxy-1,8-cineole (Fig. 2.4H) has low content in all populations, with very slight decreases in content through ontogeny.

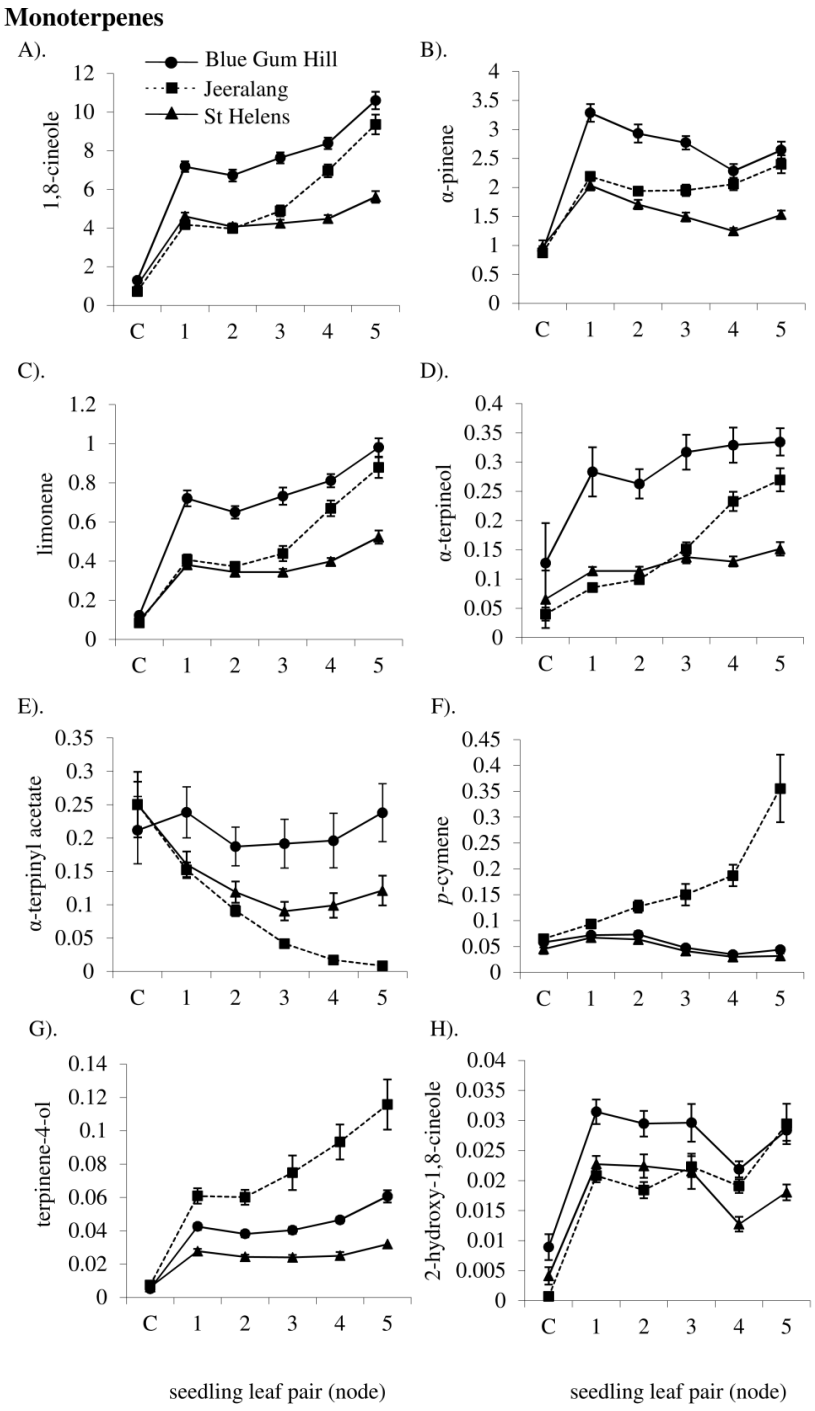

Figure 2.4(A-H). Population variation in the early ontogenetic development of eight monoterpene compounds in *E. globulus* seedlings from the cotyledon-stage (C) to node five. Least square means and standard errors are based on families represented by a bulk sample across replicates (n=10). Ontogenetic stage is represented by cotyledons and the first five consecutive true leaf pairs. 1,8-Cineole and α-pinene are expressed as mg g^{-1} DM, whilst all other compounds are expressed as equivalents of 1,8-cineole (mg g^{-1} DM). Mixed model analyses of these results are shown in Table 2.1.

The pattern of change in the sesquiterpenes is explained by both population and ontogeny as evidenced by the high percentage variation explained by these two factors (Table 2.2). The strong effect of ontogeny is reflected by the responsiveness of the Tasmanian populations to ontogeny (Fig. 2.5A-D). Jeeralang moved in the same direction, however, there were delayed levels of change in aromadendrene, alloaromadendrene, bicyclogermacrene and α-gurjunene. What is most marked in Figure 2.5 is the movement of β-caryophyllene and α-humulene in an opposing trajectory to the other sesquiterpenes (Fig. 2.5E,F). Populations expressed tighter patterns across ontogeny in these compounds. As a consequence of the opposing trajectories, there was a change in the dominance of sesquiterpene compounds throughout seedling leaf ontogeny.

Cotyledon-stage seedlings expressed none of the terpene compounds or only low levels (see Supplementary Table S2) and the paired t-test showed content to be significantly different from that the first leaf pair ($P>0.05$; Fig. 2.4A–D, G, H; Fig. 2.5), except in the monoterpenes a-terpinyl acetate and p-cymene where content was not different from that of the first true leaf pairs ($P>0.05$; Fig. 2.4E, F). Significant population differences at the cotyledon stage were only evident for 1,8-cineole, 2-hydroxy-1,8-cineole and bicyclogermacrene (Supplementary Table S2). Despite not being significant, many monoterpene compounds showed the same population ranking at the cotyledon-stage seedlings to the true leaves (Fig. 2.4A, D, F, H), but was less so in the sesquiterpenes (Fig. 2.5A, C).

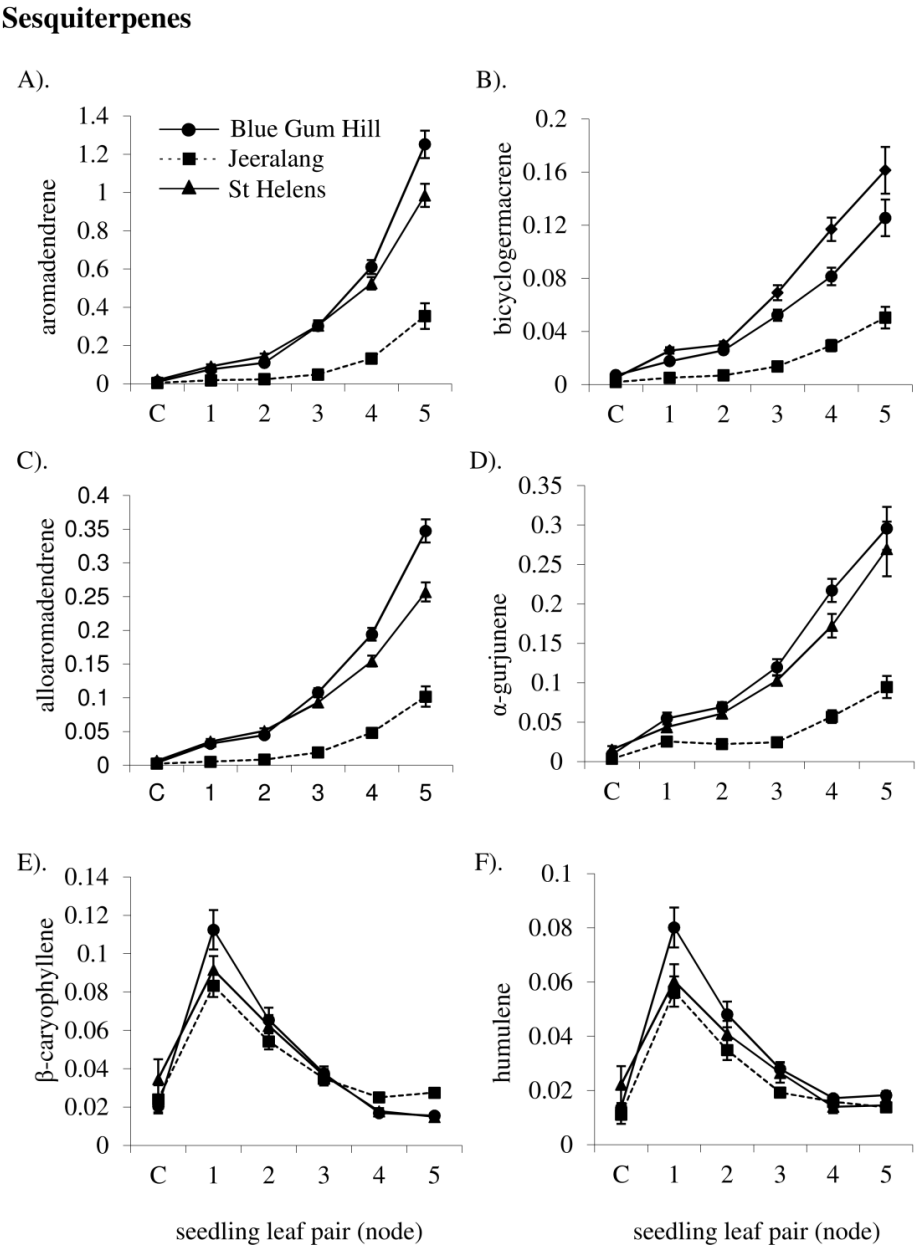

Figure 2.5(A-F). Population variation in the early ontogenetic development of six sesquiterpene compounds in *E. globulus* seedlings from the cotyledon-stage (C) to node five. Least square means and standard errors are based on families represented by a bulk sample across replicates (n=10). Ontogenetic stage is represented by cotyledons and the first five consecutive true leaf pairs. Compounds are expressed as equivalents of 1,8-cineole (mg g^{-1} DM). Mixed model analysis shown of these results in Table 2.1.

2.5 Discussion

Genetic-based ontogenetic variation in plants has been documented in morphological and reproductive traits (Wiltshire *et al.* 1998; Jordan *et al.* 1999; Dechaine *et al.* 2014) and is evident in foliar chemical traits as shown here and in other studies (Rehill *et al.* 2006; O'Reilly-Wapstra *et al.* 2007; Holeski *et al.* 2012). Whilst ontogenetic variation in foliar terpene expression has been documented in eucalypts (McArthur *et al.* 2010; Goodger *et al.* 2013a,b), this is the first study to show such variation within a genetic framework, at the early stages of eucalypt development. Further, we teased apart the effects of plant genetics, ontogenetic development and leaf age on the expression of mono- and sesquiterpene compounds in an ecologically dominant tree species. This is one of the few studies in any system to pull apart these important factors. We showed that genetic effects (at both the population and family level) and ontogeny contributed the most to terpene variation in *E. globulus* seedlings with the Tasmanian populations responding differently to the mainland Australian population. A key finding was evidence that there are differential patterns in the ontogenetic development both between and within the two major terpene classes, mono- and sesquiterpenes. Sesquiterpenes showed a more rapid response and with opposing ontogenetic trajectories in different compounds.

From the development of the first true leaves, variation was seen at both levels of the genetic hierarchy (i.e. population and family within population) with genotypes showing not only differences in absolute amounts of terpenes but also in the manner they changed through ontogeny. The genetic variation seen at the family level within each population is a significant finding as it signals the potential for the ontogenetic trajectories to evolve. These results highlight the potential for selection to act on ontogenetic patterns of change in terpene compounds, to create an ontogenetic chemical mosaic with which to defend the seedling (sensu Iason *et al.* 2011), and this can vary spatially (e.g. Singer and McBride 2012) and be under strong genetic control (Holeski *et al.* 2012).

Many of the individual compounds and the canonical discriminant analysis (terpene composition) indicate that the mainland Australian population Jeeralang express a

different ontogenetic trajectory to the two Tasmanian populations, St Helens and Blue Gum Hill. Molecular and quantitative genetic studies show that the Tasmanian *E. globulus* populations have evolved to be significantly different from mainland lineages such as represented by Jeeralang (Steane *et al.* 2006; Jones *et al.* 2013). Different chemotypes between mainland and Tasmanian populations noted in Wallis *et al.* (2011) based on adult leaf (see also O'Reilly-Wapstra *et al.* 2013b), suggest that the populations may involve quite different ontogenetic trajectories as well. In this study, the quantitative differences in the ontogenetic expression of terpenes in populations may reflect different evolutionary histories, with varying selective pressures, such as environmental conditions or levels of herbivory, at different developmental stages (Barton 2007).

Seedling stages have been shown to have important roles in community structure and dynamics and seedling defence may determine subsequent patterns of plant-herbivore interactions (Barton and Hanley 2013; Quintero and Bowers 2013). The quantitative changes demonstrated during seedling ontogeny of *E. globulus* in this study, also have the potential for such ecological consequences. For example, O'Reilly-Wapstra *et al.* (2005a) showed that a foliar terpene increase of 6.9 mg g^{-1} DM reduced brushtail possum intake. In the current study, the increase in total terpene content from the first to the fifth leaf pair (node) of seedlings exceeded this level in both Jeeralang and Blue Gum Hill populations, thus providing potential effects on mammalian herbivory within short developmental stages. Cotyledon-stage seedlings showed a lack of genetic-based variation in the majority of terpene compounds, suggesting that this developmental stage is not subject to the same diversifying selective pressures. The overall low terpene investment at the cotyledon-stage (except for α-terpinyl acetate) followed by an increase in newly emerging seedling foliage has also been documented in other eucalypt studies (e.g. Goodger *et al.* 2007; McArthur *et al.* 2010) and suggests that defence at this early developmental stage is not a priority. This is consistent with the strategy to escape the threat from herbivory by producing many small seeds for mast seedling establishment and rapid growth at this early establishment phase, rather than chemical defence (Herms and Mattson 1992; Hanley 1998; Kelly and Sork 2002). The monoterpene α-terpinyl acetate may play an important functional role in this

early growth stage because of high expression in cotyledon-stage seedlings followed by a decline through ontogeny. This role may, for example, involve defence against bacteria and fungi (Silva *et al.* 2011).

There were clear differences in the way mono- and sesquiterpenes responded to ontogeny. Overall, monoterpenes showed gradual and relatively slow increases in content, whereas sesquiterpenes were more responsive with rapid changes through ontogeny. In addition, there were clear opposing trajectories within sesquiterpenes with two compounds, β-caryophyllene and α-humulene, declining in content. Differential ontogenetic change in terpene compounds has been previously observed in *Eucalyptus*, both between (Goodger *et al.* 2013b) and within mono- and sesquiterpene classes (McArthur *et al.* 2010; Goodger *et al.* 2013a) and strongly reflects terpene biosynthetic origins. Mono- and sesquiterpenes originate from two separate biosynthetic pathways; the deoxyxylulose phosphate/methylerythritol pathway for the monoterpenes and the mevalonic acid pathway for the sesquiterpenes (Huber and Bohlmann 2004). The precursors produced in these pathways are then modified by a class of enzymes, known as terpene synthases (TPS), to produce a varied range of terpene structures that are often further modified by secondary processes to produce a diverse range of plant terpenes (Huber and Bohlmann 2004; Degenhardt *et al.* 2009; Dewick 2009). Quantitative variation in individual compounds may be determined by several factors including the number of different TPS in gene families, the fact that many TPS produce a group of products and the ability of plants to regulate the expression of TPS genes (Huber and Bohlmann 2004).

Many compounds showed tight relationships in their ontogenetic trajectories, particularly those that share a common biosynthetic precursor (Keszei *et al.* 2010), for example the monoterpenes 1,8-cineole, limonene and α-terpineol (Fig. 2.4A ,C, D); and the sesquiterpene compounds aromadendrene, alloaromadendrene, bicyclogermacrene and α-gurjunene (Fig. 2.5A-D); and β-caryophyllene and α-humulene (Fig. 2.5E, F). Across the monoterpenes (except *p*-cymene), the two Tasmanian populations expressed differences in absolute levels through ontogeny, however, their magnitude of change appeared similar, with terpene content changing

with the same trajectory. The mainland Australian population, Jeeralang, on the other hand, showed a distinct trajectory with the increases in terpene levels occurring at different rates to the Tasmanian populations. The differences in ontogenetic trajectories of the Tasmanian and the mainland Australian populations involve both mono- and sesquiterpene compounds and appears to be due to differences in the regulation of genes which control steps that occur both early and late in the biosynthetic pathways. An example is the ontogenetic increase in the content of the Jeeralang population in the sesquiterpenes aromadendrene, alloaromadendrene, bicyclogermacrene and α-gurjunene, consistently lagged behind the Tasmanian populations. The similar expression of the three populations in remaining sesquiterpenes, β-caryophyllene and α-humulene, however, suggests that the two latter compounds are not under the same genetic control as the other four. An example of different gene regulation within the monoterpene biosynthetic pathway is the observed rapid decrease in α-terpinyl acetate content in the mainland population Jeeralang. This pattern suggests a marked down regulation of a gene(s) specifically affecting its synthesis during early *E. globulus* ontogeny (Fig. 2.4E). This decline was less evident in the Tasmanian populations, particularly Blue Gum Hill. α-Terpinyl acetate is at its highest levels in the cotyledons, and the rapid decline in levels at subsequent nodes is countered by an increase in α-terpineol. α-Terpinyl acetate is derived from the acetylation of α-terpineol (see Fähnrich *et al.* 2012), a process which appears to be rapidly undertaken at the cotyledon-stage of all provenances. We suggest that this production of α-terpinyl acetate from α-terpineol may have been so rapid during this ontogenetic stage, that levels of α-terpineol remained relatively low. While the opposing pattern of ontogenetic change, particularly evident in Jeeralang, may represent a down-regulation of genes controlling this conversion, the possibility cannot be dismissed that it could be the result of back conversion of α-terpinyl acetate to α-terpineol by hydrolysation as the Jeeralang plants mature (see Dewick 2009).

The contrasting ontogenetic trajectories observed for sesquiterpenes is an important finding and demonstrates how differently compounds can behave. The sesquiterpenes with cyclopropane carbon skeletons (aromadendrene, alloaromadendrene, bicyclogermacrene and α-gurjunene; (Keszei *et al.* 2010) show a

tight relationship between compounds with an increasing ontogenetic trajectory. In contrast, the sesquiterpenes with non-cyclopropane carbon skeletons, β-caryophyllene and α-humulene (Fig. 2.5E, F) exhibited increased synthesis in the first leaf pair, followed by a rapid decline as subsequent leaves developed. Given their structural similarities, an explanation for the two apparent groupings of sesquiterpene compounds is that they are produced by at least two different sesquiterpene synthases. This result is consistent with molecular studies where QTLs for the four sesquiterpenes with positive ontogenetic trajectories were shown to be co-located on the one linkage group (O'Reilly-Wapstra *et al.* 2011). The two compounds showing a declining trajectory (β-caryophyllene and α-humulene) are known to be produced from a single sesquiterpene synthase in cotton (Yang *et al.* 2013) and hops (Wang *et al.* 2008). The remainder of compounds however, are likely to be a product of the same multi-product sesquiterpene synthase, originating from a common precursor (Keszei *et al.* 2010). Quantitative trait loci for β-caryophyllene and α-humulene have not yet been identified. This is not the first documented case of the declining patterns of β-caryophyllene and α-humulene through ontogeny, as shown in a study of terpene expression in *Salvia officinalis* (Dudai *et al.* 1999). The opposing trajectories of these two sesquiterpene groups provide evidence to support the existence of differential transcription regulation of single versus multi-product sesquiterpene synthases through *E. globulus* ontogeny. In an evolutionary context, opposing ontogenetic trajectories in terpene compounds may be a product of selection in response to herbivory or pathogen, where some compounds are more useful to defend the plant at particular stages of growth. This strategy allows plants to avoid expending resources on defence when herbivores or pathogens are absent (Simms and Fritz 1990). For example, Iason *et al.* (2011) show an invertebrate and two vertebrate herbivores to be probable selective agents at different life stages on several terpene compounds in Scots pine. Studies in other systems have shown β-caryophyllene to have roles in herbivore (Köllner *et al.* 2008; Huang *et al.* 2013) and pathogen (Sabulal *et al.* 2006; Huang *et al.* 2012) defence. From this evidence we may hypothesise that the initial high content of this compound in the first leaf pairs of *E. globulus* reflects a short-lived contribution of β-caryophyllene to defence.

In conclusion, we show diverse ontogenetic trajectories by different mono- and sesquiterpene compounds in *E. globulus* seedlings, with genetic control in ontogenetic trajectories occurring at both the population and family level. The biosynthetic pathways can explain correlated patterns of terpene change and pleiotropy involving positive and negative changes through ontogeny, with changes in the genetic-based regulation of genes appearing to occur within the different pathways. This combined evidence highlights the importance of incorporating genetics and ontogeny in studies of chemical defence. Changing ontogenetic patterns has long been recognised as a key mode of evolution in plants (Barton and Hanley 2013; Quintero *et al.* 2013; Dechaine *et al.* 2014; Hoan *et al.* 2014), including *Eucalyptus* (Hudson *et al.* 2014), but this is one of the few studies in plants (Holeski *et al.* 2012; Moore *et al.* 2014) to demonstrate a genetic basis to the different ontogenetic trajectories in chemicals associated with plant defence. The evidence presented here suggests that there exists adaptive opportunities for divergence within species to occur not only through constitutive changes in PSMs, but in ontogenetic trajectories, with the potential to create a spatio-temporal chemical mosaic for plant defence.

2.6 Acknowledgements

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Chapter 3:

Genetic stability of physiological responses to defoliation in a eucalypt and altered chemical defence in regrowth foliage

3.1 Abstract

Defoliation may initiate physiological recovery mechanisms that allow a plant to improve fitness after damage. Such responses may result in changes in plant resource allocation that influence growth and foliar chemistry. We investigated the genetic basis of variation in physiological responses including photosynthetic rates and foliar chlorophyll content, as well as the growth consequences and changes in foliar chemical content of *Eucalyptus globulus* juvenile plants to defoliation. A partial defoliation treatment that removed all upper crown leaves and the apical buds was applied to plants sourced from eight families from each of three populations representing contrasting chemical resistance to mammalian herbivory. Growth, photosynthetic rate and chlorophyll content was assessed pre-defoliation and periodically up to 12 weeks post-defoliation. Chemical content was assessed pre-defoliation, and at 12 weeks post-defoliation on the old foliage (positioned below the point of defoliation) and the new foliage of the control plants and regrowth (from axillary buds) on the defoliated plants. There were clear treatment impacts on physiological responses, growth and foliar chemical traits, although the three *E. globulus* populations did not vary in their response to foliage loss. Distinct physiological responses to defoliation were observed with treatment plants showing significant up-regulation of photosynthetic rate and increased chlorophyll content in the old foliage remaining in the lower crown. There was a significant increase in the

concentrations of a number of foliar chemical compounds in the regrowth arising from previously dormant axillary buds compared with new growth derived from apical meristems. Condensed tannins in remaining foliage 12 weeks post-defoliation were the only defence compounds to have elevated levels, although the effects of ontogeny and physiological aging could not be separated in this trial. There were changes in biomass allocation; defoliated plants had increased branching and leaf biomass, with changes in regrowth morphology to increase light capture. This study argues for multiple responses of *E. globulus* juveniles to defoliation involving apical bud loss, including elevated chemical defences matched with increased growth. These responses create an enhanced chemical mosaic to the herbivore. From a chemical defence perspective, remnant leaves after partial browsing damage are potentially more palatable than the regrowth, and this may have ecological consequences not previously taken into account in this genus.

3.2 Introduction

Defoliation by herbivores often has detrimental impacts on plant survival and fitness (O'Reilly-Wapstra *et al.* 2012). To mitigate these effects, plants respond with a range of compensatory mechanisms to allow recovery from tissue loss and damage (Haukioja and Koricheva 2000). These may include increasing photosynthetic rates in residual plant tissue (Reich *et al.* 1993; Houle and Simard 1996; Mabry and Wayne 1997; Chen *et al.* 2001; Eyles *et al.* 2011; Gori *et al.* 2014), reallocating substrates from other plant parts, increasing allocation of new photosynthates to the production of new leaves, and/or increasing the rate of cell division and elongation (Hilbert *et al.* 1981; McNaughton 1983). Mammalian browsing commonly results in loss of foliage including the removal of the apical meristems, which often activates sprouting from axillary or lateral buds. This can change host plant resource allocation (Wise and Abrahamson 2008), and subsequently alter leaf- and plant-level chemical properties (Bryant *et al.* 1991). Such changes in chemistry may be a result of induced defences, reversal of physiological aging of plant tissues, or products of altered resource allocation to secondary metabolism (Karban et al 1999), and are likely to affect plant susceptibility to future attack (Haukioja *et al.* 1990; Landsberg 1990; Paige 1992; Steinbauer *et al.* 2014).

Eucalyptus is a dominant tree genus in the Australian landscape and at different life stages is susceptible to damage from fungal pathogens (Keane *et al.* 2000), and by a range of invertebrate (Jordan *et al.* 2002; Rapley *et al.* 2004a, c) and vertebrate herbivores (Bulinski 2000; Dungey and Potts 2002). Eucalypts utilise two strategies to minimise the detrimental effects of herbivore attack. The first is to avoid damage through constitutive chemical defence (Andrew *et al.* 2007b; Henery *et al.* 2008), which has been shown to confer resistance to mammalian browsers (O'Reilly-Wapstra *et al.* 2004). The second is to initiate physiological recovery mechanisms once the damage has occurred and produce vegetative growth from dormant buds containing stored carbohydrate (Pinkard *et al.* 2007; Barry and Pinkard 2013; Burrows 2013; Eyles *et al.* 2013). Vegetative growth occurs with increasing intensity of disturbance, occurring first from the axillary buds, followed by the epicormic buds and then from the buds of the lignotubers (Burrows 2013). Following partial defoliation, plants respond with up-regulation of photosynthetic rates in the remaining leaves (Pinkard and Beadle 1998; Pinkard 2003; Pinkard *et al.* 2004; Turnbull *et al.* 2007; Barry and Pinkard 2013), and make changes in biomass partitioning, to allow compensation for loss of leaf area (Pinkard and Beadle 2000). These physiological responses are influenced by a range of variables including damage severity, growing condition and plant age (Eyles *et al.* 2013). What we do not know is whether or not juvenile stage eucalypts change their defensive chemical profile during regrowth to minimise future attacks.

In eucalypts, population divergence has been documented in numerous quantitative traits including growth (Dutkowski and Potts 1999; Stackpole *et al.* 2010), recovery response to severe defoliation (Whittock *et al.* 2003; Borzak unpub.), phase change from juvenile to adult leaves (Hamilton *et al.* 2011), reproduction (Wiltshire *et al.* 1998; Jones *et al.* 2011), and the expression of chemical defences (Jones *et al.* 2002; O'Reilly-Wapstra *et al.* 2002, Chapter 2; Rapley *et al.* 2004d; Andrew *et al.* 2007b; O'Reilly-Wapstra *et al.* 2011; O'Reilly-Wapstra *et al.* 2013b; Borzak *et al.* 2015b). However the genetic control of physiological responses to defoliation in this ecologically dominant genus, remains unexplored. Such intraspecific comparisons are important to help us gain an insight into how species may progressively adapt mechanisms to deal with herbivory (Strauss and Agrawal 1999).

Defoliation of sapling and mature eucalypts often leads to resprouts from dormant axillary buds and, in the case of eucalypts, can cause varying degrees of reversion to the juvenile phase (Wiltshire and Reid 1992; Poethig 2013). Juvenile eucalypt foliage is often more chemically defended than mature foliage on adult trees (O'Reilly-Wapstra *et al.* 2007), this is consistent with the theory that chemical defence is greater in foliage that potentially suffers more browsing pressure and has higher fitness consequence if browsed (Swihart and Bryant 2001). Severe browsing of eucalypts at the adult stage initiates regrowth from lignotubers that produce reverted juvenile coppice foliage containing higher constitutive defensive chemistry than the related adult undefoliated trees (O'Reilly-Wapstra *et al.* 2007). On the other hand, browsing at the juvenile stage may result in a carbon stress that subsequently reduces the constitutive defences of regrowth juvenile phase (Bryant *et al.* 1983). This paper addresses the gap in our knowledge of *Eucalyptus* recovery mechanisms in response to juvenile stage defoliation by investigating genetic variation in physiological mechanisms (photosynthetic rate and foliar chlorophyll content) and the chemical profile of regrowth after partial defoliation.

Eucalyptus globulus exhibits a well-studied genetic hierarchy involving race, population and family levels (Dutkowski and Potts 1999) and this provides a framework that allows quantitative traits to be investigated in an evolutionary context. In the present study, leaves of treatment plants of *E. globulus* were removed to 50% of plant height, including the apical buds. This pattern of defoliation is consistent with browsing damage by the most important mammalian herbivores of *E. globulus*, the brushtail possum (*Trichosurus vulpecula*) and the red-bellied pademelon (*Thylogale billardierii*; Bulinski and McArthur 2000). These herbivores show a preference for consuming the youngest leaves rather than stems, including the apical buds (Bulinski and McArthur 1999; McArthur *et al.* 2000). However, browsing severity may vary and occur periodically over time (Borzak *et al.* 2015a, Chapter 5). In the case of low-level disturbance, resprouting from undamaged axillary buds occurs (Burrows 2013). In this study, we investigated changes in photosynthetic rate and chlorophyll content up to 12 weeks after the defoliation treatment. Plant secondary metabolites (PSMs) were selected on the basis of known effects on mammalian (Lawler *et al.* 1999a; Wallis *et al.* 2002; O'Reilly-Wapstra *et al.* 2004) and invertebrate (Edwards *et al.*

1993; Rapley *et al.* 2008) herbivory of eucalypts. These include total terpenes, total condensed tannins, total phenolics, and two formylated phloroglucinol compounds; sideroxylonal A and macrocarpal G. Fourteen individual terpene compounds were also quantified including eight monoterpenes (including 1,8-cineole and α-pinene) and six sesquiterpene compounds. This study focused on juvenile eucalypt growth which is an important life history stage in this forest dominant. This stage is often more susceptible to herbivory, and damage at this young developmental stage may have significant impacts on later age survival (Chambers *et al.* 1996) and growth (Bulinski and McArthur 1999; Stackpole *et al.* 2010; Borzak *et al.* 2015a, Chapter 5), and therefore may be under selective pressure (O'Reilly-Wapstra *et al.* 2012).

1. Specifically I ask the following questions: Is there intraspecific genetic variation in the physiological response in juvenile stage *E. globulus* to partial defoliation?
2. If so, then how immediate is the physiological response?
3. Is there a change in leaf morphology and plant biomass allocation of defoliated plants compared to the undefoliated controls and do those changes have a genetic bases?
4. Is there a change in the defensive chemistry between the regrowth from axillary buds and new growth from apical buds in undefoliated plants and do those changes have a genetic bases?

3.3 Materials and Methods

3.3.1 Experimental design

Eucalyptus globulus has been classified into 13 genetically differentiated broad geographical groupings (Dutkowski and Potts 1999). Populations within these groups are defined as trees growing within 10km of each other (Potts and Jordan 1994). Family is progeny derived from open-pollinated seed collected from a native tree within a population. Three *E. globulus* populations were selected to encompass the chemical diversity of the species. Populations representing extremes of browsing resistance in *E. globulus* on the island of Tasmania were studied. These were the St

Helens population in the northeast of Tasmania (41° 150'S 148° 190'E) which has low foliar defensive chemistry, and Blue Gum Hill population in the southeast of Tasmania (43° 030'S 146° 520'E) which has relatively high foliar defensive chemistry (O'Reilly-Wapstra *et al.* 2004). A third population, Jeeralang North in the Strzelecki Ranges on mainland Australia (38° 190'S 146° 520'E) also has high resistance to herbivory (O'Reilly-Wapstra *et al.* 2004) and was chosen to represent a population from a different molecular lineage (Jones *et al.* 2013). Each population was represented by eight families (a single plant per family per treatment). Sample size was based on previous literature in this system (e.g. O'Reilly-Wapstra *et al.* 2005a; Barry and Pinkard 2013) by assessing the statistical power and significance findings.

Seedlings (n=48; 8 families x 3 populations x 2 treatments) were grown in a glasshouse in low nutrient potting mix containing slow release fertiliser (N:P:K 17+1.6+8.7) and at 4 months of age were put in an irrigated outdoor enclosure to allow leaves to harden. At 9 months of age, the juvenile plants were transferred to 30 cm diameter pots and left in the enclosure to establish for an additional 4 months until the commencement of the experiment. Each *E. globulus* family seedling was assigned at random to one of two experimental groups: treatment (n=24) and control (n=24). Pots were positioned in a completely randomised row by column design in the enclosure. The plants were 82.5 ± 1.2 cm (mean ± SE) in height and had an average stem diameter (at 10cm above ground level) of 0.7 cm at the start of the experiment, with an average node number of 24 ± 2 on the stem. *Eucalyptus globulus* is heterblastic but at this stage all plants were at the juvenile leaf stage, where leaves at each node are opposite and sessile. At the commencement of the experiment, the leaf pairs growing at nodes 5-6 on the lowest part of the stem had senesced naturally. To control the pattern and severity of the damage, artificial defoliation was undertaken on treatment plants. In terms of physiological response, this method is considered to be an adequate equivalent in the response generated to natural herbivory (Quentin *et al.* 2010). Following an initial assessment of physiological traits, the treatment plants were defoliated, from the crown apex downward (i.e. upper crown) to 50% the plant height (consistent with Barry and Pinkard 2013), by removing all leaves and terminal buds of the main stem and lateral branches (week 0). Care was taken not to damage

axillary buds. To achieve 50% leaf removal, leaves on the main stem positioned below the lowest lateral branches (approximately 8 leaf pairs) were left intact. The leaves on the first 2-3 nodes at the base of the lowest lateral branches were also left intact. Cotton loops with tags were attached to mark the position of defoliation. New leaves arising from axillary buds on the defoliated plants were characterised as 'regrowth'. The 24 plants not subject to defoliation served as controls. In the control plants, cotton loops with tags were attached directly below the nodes of unopened leaf buds to mark the position of new growth. All growth emerging from apical buds positioned above the cotton tags was characterised as 'new growth'. Comparisons between the upper and lower crowns of the control and treatment plants allowed key *a priori* contrasts to be made (Fig. 3.1). For the duration of the experiment, pots received adequate water (three times a day). Assessments of plant growth (height, stem diameter and lignotuber size), and physiological responses (photosynthetic rate and chlorophyll content) were made prior to defoliation treatment, then at week 1, 3, 6, 9 and 12 (6 assessments in total). Plant biomass and foliar chemistry was assessed on the harvested plants at week 12.

3.3.2 Physiological components

Light-saturated foliar photosynthesis was measured using CIRAS-1 infrared gas analyser (IRGA; PP Systems, Herts, UK) with a 2.5 cm^2 leaf chamber. The IRGA delivered CO_2 at 360 ppm and a tungsten light source delivered a photosynthetic photon flux density of 1500 mol $m^{-2}s^{-1}$. The photosynthetic rate was recorded as CO_2 uptake. Each leaf was enclosed in the chamber and left to equilibrate until a constant CO_2 flux was observed (up to 4 min). Assessments were made in fine weather conditions between 0900h and 1400h Eastern Australian Standard Time. Within the randomised row by column design, plants in a row were selected for assessment in consecutive order, but with row number and row direction chosen at random for each assessment period. Three healthy leaves of similar size and with no visible signs of aging were selected from each of the 48 plants. Selected old leaves below the cotton tags in the lower crown were assessed one day prior to the defoliation treatment at week 0, and then 1, 3, 6, 9 and 12 weeks following defoliation. At week 6 and onward, the established new growth/regrowth in the upper crown was assessed by

selecting leaves above the cotton tags. Photosynthetic assessments from week 6 were made over two consecutive days.

At every assessment time, relative chlorophyll content (based on leaf absorbance in the wavelengths 650 and 940nm) was determined on the same leaves as those analysed with the IRGA, using a Minolta SPAD-502 (Konica-Minolta, Hong Kong, China). Three measurements per leaf were made and the average was recorded. To determine chlorophyll concentration (mg cm^{-2}) the measured SPAD values were converted using a relationship between SPAD values and actual chlorophyll content determined in this study. The relationship was established from leaves sampled from treatment plants as part of the defoliation treatment at time 0, and at week 6 when three IRGA and SPAD measured leaves were picked from each crown (a total of 6 similar sized leaves from each plant) and immediately frozen to -20°C for later analysis. These leaves were not included in the final biomass measured at the end of the experiment. Chlorophyll content was quantified for each of 36 randomly selected leaves with a triple extraction method adapted from Martin *et al.* (2007). In brief, after thawing, discs were punched out of leaves and ground in a mortar with fine sand and 5 mL of liquid nitrogen. Ground leaf material was extracted with three 2 mL volumes of 100% cold acetone, centrifuged for 3 minutes then absorbance was read at 470, 645, 663 and 710 nm with a Cary UV–VIS spectrophotometer (Varian Inc., USA). Total chlorophyll (Chl a + b) was determined using equations for chlorophyll a (Chl a) and chlorophyll b (Chl b) developed by Lichtenthaler and Buschmann (2001). A relationship between SPAD values and Chl a + b concentration was established using data from the 36 leaves and applied to all SPAD values to estimate Chl a + b for all assessment time periods.

3.3.3 Growth and biomass

Plant height, stem diameter (at 10cm above ground level) and lignotuber size were measured at each assessment time. Lignotuber size was calculated as the difference between the diameter of the lignotuber (including the stem) and the diameter of the lignotuber, divided by the diameter of the stem (Whittock *et al.* 2003). Biomass harvests were conducted on the experimental plants at week 12. The cotton loops indicated the position of new growth in control plants and the position of regrowth in

the treatment plants. This was the point where the main stem and branches were cut to separate the lower and upper (new growth) crowns. Leaves, stems and branches were separated within crowns. Branch number was counted. The leaves were weighed and then bagged and frozen to -20°C for later chemical analysis. For each tree, 10 leaf samples (stratified by size) from the upper crown were analysed for Specific Leaf Area (SLA; ratio of leaf area to leaf dry mass; kg m^{-2}). Foliage from the lower crown was weighed and frozen to -20°C immediately for chemical analysis and was not available for SLA or leaf area measurements due to small sample size. All plant material, except leaves set aside for chemical analysis, was dried at 65˚C to constant mass and weighed. Dry leaf weight for chemical analysis was predicted using the linear relationship between dry and wet leaf weights of the 10 SLA leaves (R^2=0.96), and added to total dry leaf weight. Leaf area of the upper crown was determined from the relationship between dry weight and leaf area for the 10 leaves sampled. Total above ground biomass was calculated as the sum of leaves, stem and branch mass.

3.3.4 Foliar chemistry

Foliar chemistry at the beginning (week 0) and at the conclusion of the trial (week 12) was assessed in the new and old foliage from all 48 plants. To assess the change in foliar chemistry response to defoliation, the new growth (upper crown) positioned above the cotton tags in the control plants was compared to the regrowth from axillary buds in treatment plants. The chemistry of the physiologically old foliage remaining in the lower crown below the cotton tags was also determined at week 12. Assessment of the old foliage accounts for the rate of ontogenetic and developmental chemical change that may occur within the control plants, and also provides an estimate of the chemical profile of the foliage remaining on treatment plants and available to browsers (Fig. 3.1). From these samples, ten randomly selected leaves were sub-sampled and immediately frozen to -20°C for later terpene analysis. The remainder of the leaves were freeze-dried (BREDA Scientific Freeze Dryer JAVAC, Model LY-5-FM; internal temperature -35°C; pressure of 10 Pascal; heater temperature 10˚C) and then ground using a cyclotec mill. We assayed a number of PSMs including terpenes, phenolics, condensed tannins, formylated phloroglucinol compounds (sideroxylonal A and macrocarpal G). Primary metabolites nitrogen and carbon were also identified.

Fourteen individual terpene compounds were assayed including eight monoterpenes (1,8-cineole, α-pinene, limonene, α-terpineol, α-terpinyl acetate, *p*-cymene, terpinene-4-ol, 2-hydroxy-1,8-cineole) and six sesquiterpenes (aromadendrene, bicyclogermacrene, alloaromadendrene, α-gurjunene, β-caryophyllene, α-humulene).

Terpenes were assayed using thawed foliage by gas chromatography-mass spectrometry (GC-MS) following a method outlined in Borzak *et al.* (2015b, chapter 2). The concentrations of 1,8-cineole and α-pinene were expressed in milligrams per gram of Dry Matter (mg g^{-1} DM) equivalents of cineole using a 1,8-cineole standard. Total terpenes and the remaining compounds were expressed as mg g^{-1} DM cineole equivalents. The GC-MS analyses of the extracts were carried out on a Varian-450 GC with Varian 1177 split/splitless injector and Flame Ionization Detector (FID). Nine samples from the lower crown of the treatment plants did not provide sufficient leaf material for terpene analysis (4 St Helens, 3 Blue Gum Hill and 2 Jeeralang families) and were not included in the study.

Total phenolics and condensed tannins were assayed with a modified Prussian blue method for total phenolics using gallic acid standards (Graham 1992). Concentration of total phenolics was expressed as mg g^{-1} DM equivalents of gallic acid. Condensed tannins was assayed with acid butanol using purified sorghum tannin standards (Porter *et al.* 1986). Foliage for these assays was prepared and extracted following the method outlined in Hagerman (2011). Sideroxylonal A and macrocarpal G were assayed by high performance liquid chromatography following Wallis and Foley (2005). By using a pure standard of sideroxylonal A, results were expressed as mg g^{-1} DM. Results for macrocarpal G concentration were expressed as mg g^{-1} DM equivalents of macrocarpal A (using a macrocarpal A standard). Total mono- and sesquiterpene content was calculated by the sum of their individual compounds.

Freeze dried ground leaf samples were re-ground to a fine powder using a ball grinder (Retsch MM200) and analysed for nitrogen and carbon using an elemental analyser (Thermo Finnigan EA 1112 Series).

3.3.5 Statistical analysis

Assessments of photosynthetic rate and chlorophyll content of leaves in the lower and upper crown were analysed separately. These components were assessed for the effects of population, treatment and time using a repeated measures (unstructured covariance structure) mixed model fitted with PROC MIXED of SAS (SAS Institute Inc. 2009). Fixed terms in the model were population, treatment, time and all their interaction terms, with plant being the subject. Random terms were *family within population* and its various interactions with treatment and time. Biomass allocation to leaf, branch, stem and above-ground biomass was assessed with the same repeated measures mixed model but with the time variable replaced with crown.

To investigate chemical changes in response to defoliation treatment, control and treatment plants were compared within and between the upper and lower crown zones (Fig. 3.1). Foliar chemistry at the final assessment at week 12 was analysed using a repeated measures mixed model fitted with PROC MIX of SAS. Fixed terms in the model were population, treatment, crown, and all their interaction terms, with plant being the subject. Random terms were *family within population* and its interaction terms with treatment and crown. Residuals were checked for normality and homoscedasticity. Data for sideroxylonal A, macrocarpal G, terpinene-4-ol, 2-hydroxy-1,8-cineole, β-caryophyllene, humulene and α-terpinyl acetate were log transformed. For α-terpineol, the three way interaction term was excluded from the mixed model and incorporated in the error term because its inclusion did not allow model convergence.

A Bonferroni adjustment of probabilities was used to account for multiple analyses involving 17 individual compounds ($\alpha = 0{\cdot}0029$). Multiple pair-wise comparisons of significant effects were made using Tukey-Kramer adjustment. Where there was a significant treatment effect and treatment by crown interaction, *a priori* contrasts were performed in PROC MIX of SAS to test among predicted patterns of foliar chemical content on the lower and upper crowns of the control and treatment plants.

Foliar chemistry, stem diameter (at 10cm), plant height and lignotuber size at week 0, as well as leaf biomass and leaf area of the upper crown were investigated using the

same mixed model as above, but with the fixed effect of crown removed. The relationships between variables were examined using correlation models fitted with PROC CORR of SAS.

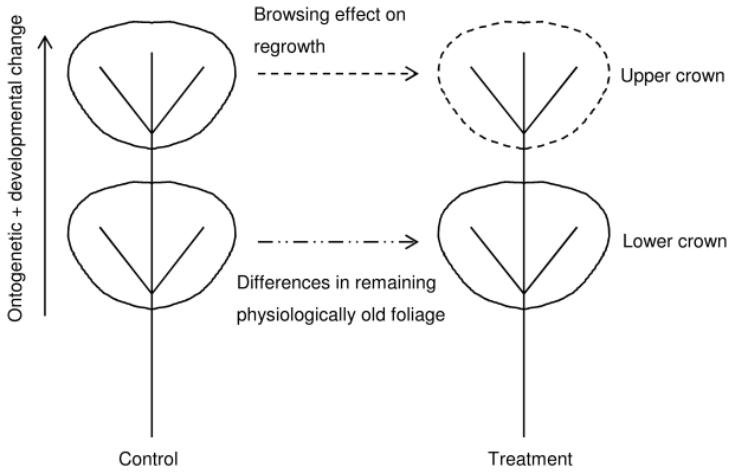

Figure 3.1. Illustration showing the lower and upper crowns with defoliation treatment and comparisons used to investigate the physiological and chemical response to a partial defoliation treatment. Key *a priori* contrasts are indicated with arrows. Changes that are due to ontogenetic and developmental age are incorporated in the change between lower and upper crowns in the control plants. The chemical change in response to the defoliation treatment in the regrowth foliage is summarised by the change in the upper crown, between the control and treatment plants.

3.4 Results

3.4.1 Photosynthetic and chlorophyll responses

The defoliation treatment plants showed significant changes in their photosynthetic rates and chlorophyll content, with upregulation and return to low levels, occurring within the 12 week time frame of the experiment (Fig. 3.2). In the days prior to defoliation at week 0, photosynthetic rate (net CO_2 uptake) and foliar chlorophyll content of the lower crown was not significantly different between plants allocated to different treatments (locality by treatment interaction $F_{2,21}$=0.4, P=0.7; Fig. 3.2). After the defoliation treatment, there was a decrease in photosynthetic rates in both the

control and treatment plants at week 1 which reflects environmental conditions on the day of assessment, however, by week 3 there was a marked difference in rates between the treatments. The remaining lower crown foliage showed a marked increase in photosynthetic rate compared to that of the control plants (treatment by time interaction $F_{2,21}$=58.5, P<0.001). Populations did not respond differently to treatments (population by treatment interaction $F_{2,21}$=0.5, P=0.6), but a difference in the absolute photosynthetic rate (population $F_{2,21}$=4.85, P=0.019; Fig. 3.2A) was due to Jeeralang having significantly higher rates than the St Helens population (P=0.005 after Tukey-Kramer adjustment for multiple comparisons). The Blue Gum Hill population generally exhibited an intermediate response. The initial response to defoliation resulted in a rapid increase in photosynthetic rates from week 1 to week 3 by 60% in Jeeralang, 66% in Blue Gum Hill and 62% in St Helens. These rates were maintained to week 6. There was no significant change in the control plants over this time period. At week 9 there was a rapid decline in photosynthetic rates in the treatment plants (treatment by time interaction $F_{5,210}$=58.5, P<0.001) with a return to the initial rates. Photosynthetic assessments in the upper crown were made in weeks 6-12, after the crown began to recover (Fig. 3.2C). The pattern in rates across assessment periods (treatment by time interaction $F_{2,84}$=4.4, P=0.02) shows photosynthetic activity was greater in the regrowth on the treatment plants than the new growth on the controls. Population ranking in the upper crowns was consistent with that in the lower crown, with Jeeralang exhibiting the highest rates. Photosynthetic rates were increased in the treatment plants (treatment $F_{1,21}$=56.1, P<0.001) with Jeeralang once again producing the highest photosynthetic rates compared to the two Tasmanian populations (population $F_{2,21}$=8.0, P=0.003; P<0.05 after Tukey-Kramer adjustment for multiple comparisons) in both controls and treatments plants.

Across the course of the experiment chlorophyll content in the lower crown showed a significant treatment effect (treatment by time interaction $F_{5,210}$= 49.55, P<0.001; Fig. 3.2B). From week 1 to week 3, chlorophyll content increased by 17% in Jeeralang, 30% in Blue Gum Hill and 31% in St Helens, whereas the control plants decreased in content by 17% in Jeeralang, 17% in Blue Gum Hill and 19% in St Helens. After week 3, chlorophyll content was stable to 6 weeks followed by a steady decline. The

control plants declined over the entire period. Despite the three populations not being significantly different in the production of chlorophyll ($F_{2,21}$=2.9, P=0.08), Jeeralang had a higher content than St Helens (P=0.04) and Blue Hill was intermediate, which is consistent with population ranking in photosynthetic rate. Chlorophyll content in the upper crown of the regrowth assessed from weeks 6-12 showed effects of population ($F_{2,21}$=4.6, P=0.02; Fig. 3.2D), treatment ($F_{1,21}$=6.0, P=0.02), and time ($F_{2,84}$=10.8, P<0.001), but not their interactions. Chlorophyll content showed the same population ranking as in the lower crown (Jeeralang > St Helens, P=0.007 after pair-wise t-test), although a clear pattern over these 6 weeks was not evident.

There were significant positive relationships between photosynthetic rate and chlorophyll content in the lower crown at all assessment periods, with the strongest association occurring from week 3, and peaking at week 6 (r=0.75, P<0.001). A weaker relationship was observed in the upper crown, again peaking at week 6 (r=0.64, P<0.001). The positive relationship between N content and photosynthetic rate at week 12 was stronger in the upper crown (r=0.74, P<0.001) than the lower crown r=0.67, P<0.001). A significant but weaker positive relationship was observed between N and chlorophyll contents in both the upper and lower crowns (R=0.47, P<0.001; R=0.66, P<0.001 respectively).

3.4.2 Growth responses

The defoliation treatment stimulated regrowth from axillary buds on the main stem and lateral branches above the cotton tags. Population differences were evident in growth and biomass traits (Tables 3.1 and 3.2), but there was no difference in the way different populations responded to the treatment. At the conclusion of the experiment at week 12, there was a 17.5% reduction in stem diameter and a 68.1% increase in shoot number in the treatment plants compared to the controls (Table 3.1). Lignotuber size was greatest in St Helens plants, which is consistent with previous studies (Whittock *et al.* 2003) and chapter 4 in this thesis, although lignotuber size, along with plant height showed no significant response to defoliation treatment.

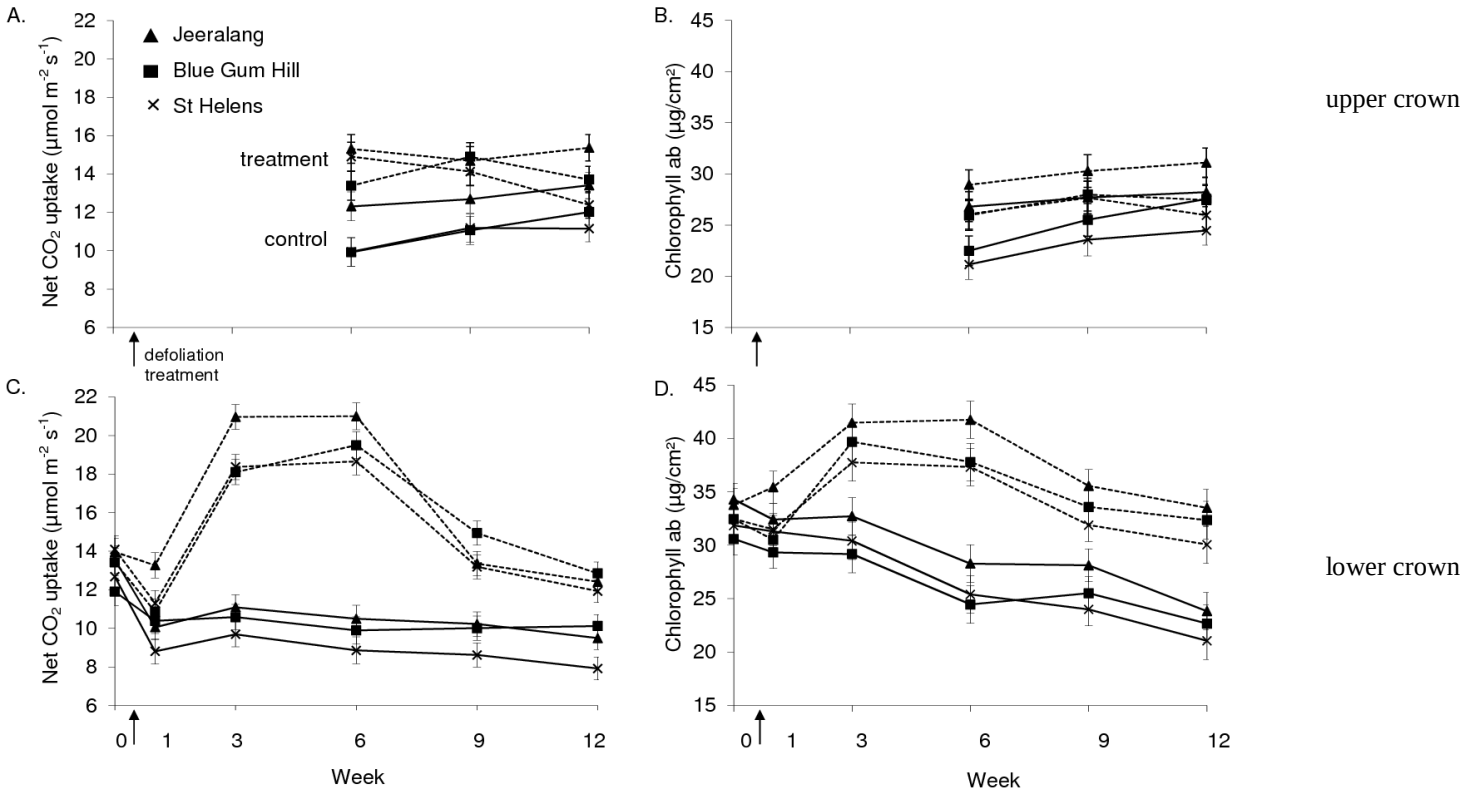

Fig. 3.2. Population variation in the photosynthetic rate (Net CO_2 uptake) and chlorophyll content (±SE) of the upper (A,B) and lower crown (new growth; C,D) in three populations of *E. globulus* plants, plotted over the course of the experiment. Control plants are indicated with solid lines, and treatment plants with dotted lines. The arrow indicates the time of defoliation treatment, one day after the initial assessment at week 0. The established new growth of the upper crown was assessed from week 6 onwards.

Table 3.1. Mean (SE) values for growth traits of *E. globulus* plants twelve weeks after a partial defoliation treatment. Also presented are the results of the mixed model analysis of treatment and genetic variation at the population level for each variable. Bold type indicates significance of the effect: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

			Population	Treatment	Population*Treatment
	Control	Defoliated plants	$F_{2,21}$	$F_{1,21}$	$F_{2,21}$
Height (cm)	98.0 (1.75)	98.58 (2.69)	1.1	0.0	0.1
Stem diameter (mm)	10.52 (0.17)	8.68 (0.14)	0.5	**126.0*** **	0.1
Shoot number	11.92 (0.74)	20.04 (1.77)	0.2	**17.2*** **	0.8
Lignotuber size	0.78 (0.07)	0.77 (0.07)	**9.2*** **	0.0	0.5

Table 3.2. Mean (SE) values for biomass allocation to above ground tissue of *E. globulus* plants 12 weeks after a 50% defoliation treatment. Values are expressed as grams dry matter (g DM). The results of the repeated mixed model analysis for the effect of population, treatment and crown (lower and upper) are also presented. Where there are significant treatment effects, different letters indicate significant differences between crowns in the control and defoliated plants after pair-wise comparison, $P < 0.05$.

						Population	Treatment	Crown	Crown* Treatment	Population* Treatment	Population *crown	Population *Crown* Treatment
	Crown	Control		Defoliated plants		$F_{2,21}$	$F_{1,21}$	$F_{1,21}$	$F_{1,21}$	$F_{2,21}$	$F_{2,21}$	$F_{2,21}$
Leaf	Upper	12.19 (0.66)	b	13.90 (0.57)	c	**5.9****	**127.8*****	**83.1*****	**119.0*****	1.0	2.3	1.1
	Lower	13.11 (0.51)	bc	3.61 (0.29)	a							
Branch	Upper	1.80 (0.09)		1.69 (0.14)		0.7	0.2	3.0	1.3	0.7	2.1	0.1
	Lower	1.89 (0.17)		2.14 (0.21)								
Stem	Upper	0.65 (0.06)	b	0.22 (0.06)	a	0.2	**44.0*****	**996.5*****	**30.1*****	0.01	0.2	0
	Lower	21.92 (0.88)	d	15.19 (0.72)	c							
Above-ground	Upper	14.65 (0.59)	a	15.82 (0.68)	a	1.3	**74.5*****	**239.0*****	**93.7*****	0.1	1.9	0.5
biomass	Lower	36.92 (1.39)	c	20.94 (0.85)	b							

Biomass allocation to above-ground tissue following defoliation also did not differ among populations (Table 3.2), and allocations to the lower and upper crowns were identified using *a priori* contrasts ($P<0.05$). As a result of the defoliation treatment, there were the expected reductions in leaf and above-ground biomass in the lower crown. Interestingly, stem biomass of the lower crown was also reduced. In the upper crown there was a 66% decrease in stem biomass, but there was no change in above ground biomass. Leaf biomass of the regrowth was 5.1% greater than the new growth in the control plants. This reflects a change in resource allocation from stem to leaves, with evidence of overcompensation with greater biomass of regrowth foliage. The regrowth on treatment plants exhibited increased SLA i.e. they were bigger and thinner than new growth on the controls. Mean leaf area of regrowth on the treatment plants (1.70 ± 0.09 m^2) was 32% greater ($F_{1,21}=19.5$, $P<0.001$) than the leaf area of new growth on the control plants (1.29 ± 0.07 m^2). There was a significant population difference ($F_{2,21}=12.9$, $P<0.001$) for this trait, with St Helens exhibiting greater leaf area compared to the other populations. There was no significant interaction between population and treatment in SLA ($F_{2,21}= 1.5$, $P=0.25$), although a significant population difference ($F_{2,21}= 6.4$, $P=0.007$) showed St Helens with the highest value compared to the other populations. The mean SLA of the regrowth in the defoliated plants (1.22 ± 0.28 $m^2\,kg^{-1}$) was 15% greater than the new growth of the control plants (1.06 ± 0.24 $m^2\,kg^{-1}$).

3.4.3 Foliar chemical responses

Foliar chemical composition showed clear differences among the populations prior to the defoliation treatment (week 0), with the Tasmanian populations (Blue Gum Hill and St Helens) being more similar to one another than to the Australian mainland population Jeeralang (data not shown). Mixed model analysis followed by Bonferroni adjustment ($P<0.0029$) identified significant effects of crown, treatment, population and their interactions on the chemical content of plants 12 weeks after the defoliation treatment. The effect of crown followed by the crown by treatment interaction was the largest effect on most of chemical components in this study (Table 3.3). The change in chemistry between the lower and upper crowns of the control plants reflects the effects of ontogenetic and physiological aging.

Table 3.3. Results of the mixed model analysis examining genetic variation and changes in foliar compounds in *E. globulus* juvenile plants 12 weeks after a partial defoliation treatment. Total mono- and sesquiterpene content was calculated as the sum of their individual compounds. Individual terpene compounds are listed in order of dominance within classes. Significance of effects: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bold type indicates significance ($P < 0.0029$) after the Bonferroni adjustment. *Family within population*, and its interaction with treatment and crown were used as the error terms to test the effects shown. *Family within population* was significant only in 1,8-cineole (Z=3.4, P<0.001), *p*-cymene (Z=2.0, P=0.02) and terpinene-4-ol (Z=2.7, P<0.001). DDF = denominator degrees of freedom associated with the random error terms used to test the fixed effects. The convergence of some traits required the removal of random effect terms resulting in high DDF values. [a] indicates log transformed.

	DDF	Population	Treatment	Crown	Treatment* Population	Treatment* Crown	Crown* Population	Crown*Treatment* Population
Numerator df		2	1	1	2	1	2	2
Nitrogen	20	0.5	**23.1*** **	**42.9*** **	0.16	7.7*	0.6	5.6*
Carbon	20	**16.0*** **	**27.5*** **	**203.8*** **	0.44	**22.9*** **	2.0	0.7
Total phenolics	20	6.7**	1.2	0.2	0.65	**16.1*** **	**15.2*** **	1.1
Condensed tannins	20	5.4*	0.1	**124.7*** **	1.89	**26.0*** **	0.6	0.0
Sideroxylonal [a]	20	7.3**	0.4	**419.3*** **	0.71	**151.6*** **	0.4	0.8
Macrocarpal G [a]	20	**11.1** **	0.8	**155.7*** **	0.03	9.9**	1.5	1.8
Total terpene	12	**20.2*** **	0.0	**413.6*** **	2.07	**34.8*** **	**14.5*** **	1.4
Total monoterpenes	12	**18.5*** **	1.1	**441.3*** **	1.28	**63.5*** **	**16.9*** **	2.2
Total sesquiterpenes	12	7.1**	0.8	**152.4*** **	1.06	0.1	5.8*	0.00
Monoterpenes								
1,8-Cineole	12	**14.9*** **	1.3	**412.8*** **	1.01	**57.5*** **	**15.8*** **	2.3
α-Pinene	12	**13.1** **	0.8	**254.8*** **	0.38	**42.9*** **	8.1**	1.2
Limonene	12	8.6**	0.1	**328.7*** **	2.70	**38.1*** **	**13.9*** **	0.5
α-Terpineol	21	**14.1*** **	0.7	**242.1*** **	3.07	**50.5*** **	**12.4*** **	0.9
p-Cymene	12	**10.6** **	7.8	7.8*	6.27*	0.5	4.7*	1.4
α-Terpinyl acetate [a]	12	3.4	0.2	0.1	0.54	1.4	0.3	1.2
Terpinene-4-ol [a]	12	**30.9*** **	**65.5*** **	**102.0*** **	5.49*	**60.7*** **	0.6	0.8
2-Hydroxy-1,8-cineole [a]	12	7.5**	**49.3*** **	**77.1*** **	0.77	0.1	0.5	6.5
Sesquiterpenes								
Aromadendrene	12	7.8**	2.3	**138.5*** **	0.93	0.0	6.4*	0.0
Alloaromadendrene	12	8.5**	0.6	**158.9*** **	0.72	2.1	6.2*	0.1
Bicyclogermacrene	12	3.5	0.8	**85.5*** **	1.74	0.9	2.2	0.4
α-Gurjunene	12	6.1*	0.0	**184.8*** **	1.27	0.0	9.2**	0.7
β-Caryophyllene [a]	12	**14.3*** **	13.7**	**86.1*** **	0.72	**21.7*** **	3.7	1.6
α-Humulene [a]	12	4.3	**19.0*** **	**196.2*** **	1.24	**33.0*** **	5.4*	2.2

In the control plants, all compounds showed greater content in the upper crown than the lower crown except condensed tannins which, was greater in the lower crown, and total phenolics, which showed no change. Despite this, there was no significant three way interaction, indicating a similar change in chemistry in the three *E. globulus* populations. The crown by treatment interaction reflected the difference in foliar chemistry of the regrowth (from axillary buds) in the treatment plants compared to the new growth (apical) of the undefoliated controls. Following Bonferroni adjustment, this crown by treatment interaction was statistically significant in carbon, total phenolics, condensed tannins, sideroxylonal A, total terpenes, total monoterpenes and seven individual mono- and sesquiterpene compounds (Table 3.3).

The smaller effect of crown by population interaction on total phenolics, total terpene, total monoterpenes, 1,8-cineole, limonene, α-terpineol and β-caryophyllene resulted from differences in the magnitude of change that occurred between the lower and upper crowns, but all populations changed in the same direction. Pair-wise t-tests for specific contrasts indicated the direction of chemical content change in response to the defoliation treatment (Tables 3.4a,b). Chemical upregulation in the regrowth of the treatment plants compared to the new growth in control plants was evident in a number of compounds, with increases in: nitrogen (21%), sideroxylonal A (37%), total terpene (15%), total monoterpenes (25%), 1,8-cineole (24%), α-pinene (30%), limonene (21%), α-terpineol (9%) and 2-hydroxy-1,8-cineole (94%). On the other hand, the content of carbon, total phenolics and condensed tannins was reduced in the new growth foliage by 0.5%, 6.8% and 39%, respectively. The remaining significant treatment by crown effects were products of a change in the chemical profile of the leaves remaining in the lower crown positioned below the cotton tags (Tables 3.4a,b). Elevated levels were detected in N and condensed tannins, while carbon, sideroxylonal A, total terpene, total monoterpenes, 1,8-cineole, limonene, terpinene-4-ol, β-caryophyllene and α-humulene decreased in content.

Table 3.4a. Summary of mean content (SE) of chemical compounds determined for *E. globulus* plants (n=48) within lower and upper crown, 12 weeks following defoliation treatment. Total monoterpene and sesquiterpene (Table 3.4b) content was calculated by the sum of their individual compounds. Individual terpene compounds are listed in order of dominance within classes. Different letters indicate significant differences in chemical content between upper and lower crowns in the control and defoliated plants (pair-wise contrasts (t-test), $P < 0.05$) for each separate compound.

	Units	Crown	Control		Defoliated plants	
Nitrogen	% DM	Lower	0.72 (0.02)	a	0.81 (0.02)	bc
		Upper	0.78 (0.03)	b	0.94 (0.02)	d
Carbon	% DM	Lower	43.79 (0.14)	b	42.65 (0.22)	a
		Upper	44.78 (0.14)	d	44.57 (0.14)	c
Total phenolics	mg g^{-1} DM	Lower	90.19 (2.00)	ab	91.76 (2.28)	bc
		Upper	93.36 (2.56)	bc	87.04 (2.51)	a
Condensed tannins	mg g^{-1} DM	Lower	17.78 (0.96)	c	23.09 (2.09)	d
		Upper	11.77 (1.13)	b	7.24 (0.53)	a
Sideroxylonal A	mg g^{-1} DM	Lower	2.64 (0.29)	b	1.78 (0.26)	a
		Upper	3.69 (0.38)	c	5.05 (0.56)	d
Macrocarpal G	Macrocarpal A eqivalents (mg g^{-1} DM)	Lower	2.98 (0.31)	ab	2.06 (0.26)	a
		Upper	5.01 (0.52)	c	5.09 (0.56)	bc
Total terpene	Cineole equivalents (mg g^{-1} DM)	Lower	37.20 (2.82)	b	27.42 (3.41)	a
		Upper	60.17 (3.97)	c	69.24 (4.08)	d
Total monoterpenes	Cineole equivalents (mg g^{-1} DM)	Lower	24.26 (2.02)	b	17.96 (2.34)	a
		Upper	36.15 (2.74)	c	45.06 (2.86)	d
Total sesquiterpenes	Cineole equivalents (mg g^{-1} DM)	Lower	3.12 (0.31)	a	2.17 (0.58)	a
		Upper	6.93 (0.69)	b	6.30 (0.78)	b
<u>Monoterpenes</u>						
1,8-Cineole	mg g^{-1} DM	Lower	16.56 (1.41)	b	12.15 (1.59)	a
		Upper	24.71 (1.86)	c	30.64 (2.10)	d
α-Pinene	mg g^{-1} DM	Lower	4.95 (0.45)	a	4.08 (0.59)	a
		Upper	7.28 (0.63)	b	9.49 (0.70)	c
Limonene	Cineole equivalents (mg g^{-1} DM)	Lower	1.57 (0.15)	b	0.87 (0.15)	a
		Upper	2.66 (0.23)	c	3.22 (0.24)	d
α-Terpineol	Cineole equivalents (mg g^{-1} DM)	Lower	0.55 (0.07)	a	0.35 (0.09)	a
		Upper	0.86 (0.11)	b	1.17 (0.12)	c
p-Cymene	Cineole equivalents (mg g^{-1} DM)	Lower	0.30 (0.09)	cd	0.29 (0.12)	b
		Upper	0.26 (0.10)	abc	0.18 (0.06)	a
α-Terpinyl acetate	Cineole equivalents (mg g^{-1} DM)	Lower	0.14 (0.06)	a	0.07 (0.04)	a
		Upper	0.21 (0.10)	c	0.17 (0.09)	a
Terpinene-4-ol	Cineole equivalents (mg g^{-1} DM)	Lower	0.14 (0.02)	b	0.06 (0.01)	a
		Upper	0.15 (0.02)	b	0.14 (0.02)	b
2-Hydroxy-1,8-cineole	Cineole equivalents (mg g^{-1} DM)	Lower	0.04 (0.00)	b	0.08 (0.01)	c
		Upper	0.02 (0.00)	a	0.03 (0.00)	b

Table 3.4b. Summary of mean content (SE) of sesquiterpene compounds determined for *E. globulus* plants (n=48) within lower and upper crown, 12 weeks following defoliation treatment. Individual compounds are listed in order of dominance within classes. Different letters indicate significant differences in chemical content between crowns in the control and defoliated plants (pair-wise contrasts (t-test), $P < 0.05$).

Sesquiterpenes	Units	Crown	Control		Defoliated plants	
Aromadendrene	Cineole equivalents (mg g^{-1} DM)	Lower	2.04 (0.21)	a	1.39 (0.33)	a
		Upper	3.99 (0.41)	b	3.34 (0.44)	b
Alloaromadendrene	Cineole equivalents (mg g^{-1} DM)	Lower	0.54 (0.05)	a	0.35 (0.08)	a
		Upper	0.97 (0.10)	b	0.92 (0.11)	b
Bicyclogermacrene	Cineole equivalents (mg g^{-1} DM)	Lower	0.27 (0.03)	a	0.24 (0.11)	a
		Upper	0.90 (0.11)	b	1.03 (0.15)	b
α-Gurjunene	Cineole equivalents (mg g^{-1} DM)	Lower	0.20 (0.03)	a	0.17 (0.08)	a
		Upper	0.94 (0.11)	b	0.88 (0.11)	b
β-Caryophyllene	Cineole equivalents (mg g^{-1} DM)	Lower	0.04 (0.01)	b	0.01 (0.00)	a
		Upper	0.07 (0.01)	c	0.07 (0.01)	cb
α-Humulene	Cineole equivalents (mg g^{-1} DM)	Lower	0.03 (0.00)	b	0.01 (0.00)	a
		Upper	0.05 (0.00)	c	0.06 (0.00)	c

The comparisons involving the differences in the remaining lower crown leaves sampled were confounded by differences in several factors. Those sampled on the treatment plants were on average closer to ground level, ontogenetically younger and physiologically older (see Borzak *et al.* 2015b, chapter 2), than the remaining control leaves. Thus any change in content in response to the treatment in the lower crown may be either a response to the treatment or a consequence of ontogeny and/or physiological ageing. Regardless, remaining leaves in the lower crown were clearly different on average compared to upper crown in both treatment and control plants and so may represent a change in food source quality for browsing.

3.5 Discussion

The four key results of this research are: 1) the genetic stability of all assessed response traits to partial defoliation among *E. globulus* populations; 2) immediate and dramatic upregulation of photosynthetic rates in the foliage remaining on plants within 3 weeks after defoliation; 3) changes in leaf morphology (determined by SLA) and plant biomass allocation after defoliation; and 4) changes in foliar chemical composition in regrowth of defoliated plants compared to the new growth in control plants.

3.5.1 Genetic stability of physiological recovery mechanisms

While differences in chemical profiles found in this study are consistent with well-documented differences between these *E. globulus* populations (O'Reilly-Wapstra *et al.* 2004; O'Reilly-Wapstra *et al.* 2013b; Borzak *et al.* 2015b, Chapter 5), the genetic basis of photosynthetic responses and foliar chlorophyll content has not been previously reported. Here we showed that despite observed population differences in physiological, chemical, and biomass allocation traits, the three populations responded in the same ways to the defoliation treatment. Plant recovery traits and foliar chemical changes that occur in response to defoliation and are under selection exerted by herbivores are often genetically determined and show heritable genetic variation (Baucom and Mauricio 2008; Gols *et al.* 2008; Muola *et al.* 2010; Carmona and Fornoni 2013). The populations used in the current study are genetically different, widely geographically separated and occupy different environments (Dutkowski and Potts 1999; Jones *et al.* 2013). In an eco-evolutionary setting, the absence of population differences in the physiological, growth and chemical response to severe loss of biomass suggests that the strength and consistency of selective forces by these herbivores would be maintained across environments. The extent of intraspecific variation may vary with plant life-history stage, type of damage, herbivore type and the timing of the assessment relative to the occurrence of the damage (Muola *et al.* 2010). Homogeneity of intraspecific variation should always been questioned if subtle differences between populations exist, as they may be detected with a larger sample size (Muola *et al.* 2010). Studies of genetic stability in eucalypt response to abiotic stresses have shown mixed results, for example conservative chemical and growth traits were observed in responses to elevated carbon dioxide in *E. globulus* and *E. pauciflora* (McKiernan *et al.* 2012). In contrast, a recent study examining the effects of varying levels of water availability on growth, leaf morphology and physiology in *E. tricarpa* showed responses among provenances differed indicating a capacity for this species to adapt to a changing environment (McLean *et al.* 2014).

3.5.2 Photosynthetic upregulation and increased chlorophyll content in response to defoliation

Increased photosynthetic rates in the remaining leaves is not an uncommon response in eucalypts following natural (Quentin *et al.* 2010) and artificial (Pinkard and Beadle 1998; Pinkard *et al.* 1998; Medhurst and Beadle 2005; Quentin *et al.* 2012; Barry and Pinkard 2013; Lisboa *et al.* 2014) defoliation. It is also consistent with the response to defoliation in other woody tree species, such as conifers (Reich *et al.* 1993; Chen *et al.* 2001; Lavigne *et al.* 2001; Eyles *et al.* 2011), acacia (Medhurst *et al.* 2006) and poplar (Maurin and DesRochers 2013). In the current study, both foliar nitrogen and chlorophyll content showed a strong relationship to photosynthetic rates, which is consistent with physiological mechanisms at the leaf-level (Trumble *et al.* 1993; Quentin *et al.* 2010). The strength and persistence of photosynthetic rate and chlorophyll production in eucalypts may vary depending on a number of factors, including leaf age (Lisboa *et al.* 2014), growing conditions, the extent of damage (Barry and Pinkard 2013), the age of the plant at the time of defoliation and the time of assessment following defoliation (Eyles *et al.* 2013). The strong and transient responses, as observed in this study, are typical of defoliated eucalypt trees involving removal of apical buds at the juvenile stage (Quentin *et al.* 2012; Barry and Pinkard 2013).

3.5.3 Altered leaf biomass and area in response to defoliation

Eucalypts globulus plants in this study were not only able to fully recover (in terms of above ground biomass) from partial defoliation in the twelve weeks after damage, but they overcompensated with a greater regrowth biomass (arising from axillary buds) than the new growth in the control plants. Overcompensation is an important recovery strategy in woody plants in other systems (Fornoni 2011; Zhao and Chen 2012; O'Reilly-Wapstra *et al.* 2014b), and whilst eucalypts are known for their ability to recover from loss of biomass (Pinkard *et al.* 2006b; Borzak *et al.* 2015a) through vegetative regeneration (Burrows 2013), only a few studies have shown overcompensation in this genus (e.g. Barry *et al.* 2012). Following defoliation, the morphology of the new foliar growth was markedly different, with leaves growing

larger and thinner (as determined by SLA) or less dense (Vile *et al.* 2005; this trait was not assessed) compared to the growth in the upper crown of the control plants. Although previous studies do not show consistent responses of SLA to defoliation, increasing leaf area is a well-documented recovery strategy utilised by eucalypts to promote the capture of light in response to defoliation (Barry and Pinkard 2013; Eyles *et al.* 2013). The overall impact of partial defoliation on *E. globulus* juveniles is a sacrifice in structural allocation, with a reduction in resources to the stem, whilst increasing leaf area to maximise photosynthetic area. Changes in the patterns of biomass allocation often come at the expense of stem and root growth (Eyles *et al.* 2009; Quentin *et al.* 2011a). Root biomass cannot be accounted for in this study as it was not assessed.

3.5.4 Altered foliar chemistry in response to defoliation

Since new growth is often favoured by herbivores compared to mature leaves, we might expect preferential allocation of chemical defence to the vulnerable tissue of new foliage. Indeed, the new growth of the control plants did show increased chemical defence, but the regrowth in the treatment plants became even more heavily defended. A number of chemical compounds assessed in this study showed elevated levels in the regrowth from axillary buds, including nitrogen, sideroxylonal A, total terpene, total monoterpene, 1,8-cineole, α-pinene, limonene, a-terpineol and 2-hydroxy-1,8-cineole. The similar pattern of change between compounds reflect previously documented chemical correlations in eucalypt foliage, such as sideroxylonal A and terpenes (Moore *et al.* 2004; Andrew *et al.* 2005), as well as known biosynthetic origins. For example, individual terpenes that are produced by a common precursor such as the monoterpenes 1,8-cineole, α-pinene, limonene, a-terpineol and 2-hydroxy-1,8-cineole (Keszei *et al.* 2010) responded similarly to the treatment. These results demonstrate the significant shifts that can occur after defoliation involving removal of apical buds, with regrowth from axillary buds being better defended than the new growth in related undefoliated plants. The expression of PSMs may vary dramatically through ontogenetic development, with changes occurring between and within plant life stages of many plant species (Barton and Koricheva 2010; Quintero and Bowers 2012; Goodger *et al.* 2013a; Moore *et al.*

2014), including in *E. globulus* (O'Reilly-Wapstra *et al.* 2007; Borzak *et al.* 2015b, chapter 2). This is consistent with the results of the current study, where the combined effects of ontogeny and physiological ageing altered the chemical profile of the lower and upper crowns of the control plants. In terms of development, regrowth leaves from axillary buds are ontogenetically older but physiologically younger than comparable node leaves, such that the regrowth from dormant axillary buds reverts to a more juvenile form (Wiltshire and Reid 1992; Poethig 2013). I would therefore expect the chemical content of the regrowth to tend towards the lower levels found in ontogenetically younger but physiologically older leaves (lower crown of the control plants). From a chemical perspective, the chemical profile of regrowth is not consistent with the reversion to an ontogenetic earlier stage, but is consistent with plant allocation of more chemical resources to defend overcompensation regrowth. This evidence supports the theory that herbivores are acting as selective agents on PSMs (Mauricio and Rausher 1997), with the more susceptible *E. globulus* regrowth becoming more defended. This strategy provides a chemical mosaic to the herbivore, with the remnant leaves potentially more palatable than the regrowth. Increases in primary compounds such as nitrogen in the regrowth may however, alter foliage quality sufficiently to override secondary metabolite defence compounds and instead attract herbivory. For example, Steinbauer *et al.* (2014) demonstrated that young, morphologically juvenile leaves produced from epicormic buds after defoliation provided higher quality foliage with greater nitrogen, and were subsequently more palatable to the invertebrate *Psyllaephagus spp*.

Condensed tannins are known to play an important role in deterring feeding by invertebrate (Lindroth and Hwang 1996; Rapley *et al.* 2008; Barbehenn and Peter Constabel 2011) and marsupial herbivores (Harborne 1991; O'Reilly-Wapstra *et al.* 2005a), yet condensed tannins, as well as total phenolics, were the only compounds to show reduced concentrations in the regrowth from axillary buds. This is consistent, however, with the dramatic drop to non-detectable levels of condensed tannins in juvenile foliage in a study that compared the chemistry of one year old *E. globulus* coppiced plants to related adult plants (O'Reilly-Wapstra *et al.* 2007), and supports hypotheses that those compounds evolved for other purposes than for herbivore defence (Hagerman *et al.* 1998; Close and McArthur 2002). Reduced condensed

tannins and total phenolics in regrowth foliage has been documented in other woody trees, including *Acacia spp, Metrosideros umbellate,* and *Combretum apiculatum* (Du Toit *et al.* 1990; Kuhajek *et al.* 2006; Fornara and Du Toit 2007; Rooke and Bergström 2007). Such changes may be due to carbon stress (Bryant *et al.* 1983), but they have been also associated with altered source:sink ratios whereby regrowth demands increased carbohydrate, which in turn reduces concentrations of metabolic end products such as lignin and phenols, including condensed tannins (Du Toit *et al.* 1990; Bryant *et al.* 1991).

Condensed tannins were the only PSM to have elevated levels in the remaining physiologically old leaves of the lower crown, and are a strong candidate for induction following damage in eucalypts (Barry *et al.* 2001; Eyles *et al.* 2003a; Rapley *et al.* 2008) and in other systems (Feeny 1970; Macauley and Fox 1980; Cooke *et al.* 1984; Erwin *et al.* 2001; Fritz *et al.* 2001; Osier and Lindroth 2001, 2004; Boege 2005b; Schweitzer *et al.* 2008; Holeski *et al.* 2012). However, the effects of ontogeny and physiological aging on PSM content cannot be separated in the present study. The leaves sampled from the defoliated plants were on average ontogenetically younger but physiologically older than those sampled from the control plants. An induced response would be supported if condensed tannin content increased with ontogeny and decreased with leaf aging. Few studies have explored ontogenetic change in condensed tannins in eucalypts (O'Reilly-Wapstra *et al.* 2007), and studies in other systems have shown inconsistent results with condensed tannins being either the same or high in both physiologically young and old foliage, or varying according to season.(Macauley and Fox 1980; Cooke *et al.* 1984; Erwin *et al.* 2001).

In conclusion, this study demonstrates the dynamic nature of the expression of foliar defence compounds after defoliation, and contributes to the better understanding of recovery mechanisms in *E. globulus*. The results suggest that juvenile *E. globulus* trees utilise multiple independent strategies to respond to partial defoliation. Plants increased leaf area at the expense of structural allocation to stem to maximise photosynthetic area, as well as upregulating photosynthesis, and boosting defence in regrowth from axillary buds. There is a growing literature that suggests growth and

chemical defence may evolve simultaneously within a population, coexisting as complementary rather than competing alternatives (Rosenthal and Kotanen 1994; Rausher 1996; Strauss and Agrawal 1999; Stowe *et al.* 2000; Rausher 2001; Carmona and Fornoni 2013), which may allow mixed strategies to defend against multiple stresses, such as multiple herbivores (Wise and Rausher 2013) and/or abiotic stresses.

3.6 Acknowledgements

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Chapter 4:
The survival and recovery of *Eucalyptus globulus* seedlings from severe defoliation

4.1 Abstract

Catastrophic damage that occurs at the early establishment stage of eucalypts, such as by browsing mammals, may have deleterious effects on survival, growth and tree shape. An important recovery strategy of many eucalypts is resprouting from basal storage buds (lignotubers) but until now, intraspecific variation in recovery and the consequence of seedling resource allocation to storage of carbohydrate reserves has been relatively unexplored. Identifying such variation in recovery traits is important for our understanding of how plants adapt to severe defoliation, and the extended effects on communities and ecosystems.

Here we investigated genetic variation in recovery following a single severe defoliation (decapitation) treatment at the early establishment stage of two *Eucalyptus globulus* populations from the island of Tasmania. The selected populations had differing expressions of foliar defences, and apparent differential resource allocation to storage. Trees of the Blue Gum Hill population from the wet southern forests of Tasmania produce small lignotubers but have comparatively high foliar defences, whereas St Helens trees from the dry forests in north-eastern Tasmania have relatively well developed lignotubers and low foliar defences. Seedlings of ten families from each population were subjected to two artificial decapitation treatments in the nursery at 6 months old, planted in 10 replicates in the field 5 months after treatment, and assessed up to 32 months after treatment.

The results showed differential survivorship between the two populations following severe defoliation, suggesting that adaptive opportunities exist for increased sprouting ability between *E. globulus* populations. The difference in sprouting ability between these populations was consistent with differences in lignotuber size and stem base diameter. The surviving plants exhibited genetic stability not only in constitutive differences, but in the way they changed in response to decapitation treatment. After 20 months of growth in the field, there were no lasting effects on important growth traits, with population differences overriding the initial treatment effects. These results are consistent with differential resource allocation of *E. globulus* populations that have adapted to wet or dry environments.

4.2 Introduction

Resprouting is a key strategy for many plants faced with unavoidable disturbance or damage, such as fire, wind or herbivory (Karban and Baldwin 1997; Bond and Midgley 2001). Like many plant traits, resprouting can exhibit inter- and intraspecific variation and identifying such variation will not only help our understanding of the evolutionary processes that shape recovery strategies in plants (Bond and Midgley 2003; Moreira *et al.* 2012; He 2014; Shibata *et al.* 2014; Aparicio *et al.* 2015), but also inform how the long-term consequences of resprouting may affect plant population dynamics (Bond and Midgley 2001) and associated community assemblages (Hrabar and Du Toit 2014).

Eucalypts are renowned for their capacity to recover from catastrophic damage by producing coppice (Noble 2001; Noble and Diggle 2014). High intensity biomass loss initiates re-sprouting from an organ at the base of the trunk called a lignotuber that is comprised of dormant buds (Vesk and Westoby 2004; Burrows 2013). These structures are related to the axillary buds in the cotyledonary axils and the axils of first few true leaves, and serve as a large source of stored bud linked with roots that supply stores of non-structural carbohydrates (Burrows 2013; Clarke *et al.* 2013). Such recovery mechanisms are important particularly during early establishment as damage during this vulnerable stage may have significant fitness costs to the plant, such as reduced survival and seedling growth rate, and altered tree shape (Bulinski 1999; Close *et al.* 2010; O'Reilly-Wapstra *et al.* 2012; Borzak *et al.* 2015a, Chapter

5). The extent of eucalypt recovery may depend on timing, frequency, severity and pattern of defoliation (Noble 2001; Pinkard 2003; Wills *et al.* 2004; Pinkard *et al.* 2006b; Noble and Diggle 2014).

Eucalypts are noted for their high level of intraspecific genetic variation in morphological, reproductive and chemical traits (Potts and Wiltshire 1997; Hamilton *et al.* 2011; Jones *et al.* 2011; O'Reilly-Wapstra *et al.* 2014a; Padovan *et al.* 2014; Borzak *et al.* 2015b, Chapter 2). Sprouting from enlarged lignotubers is no exception, exhibiting genetic-based variation in several species (Walters *et al.* 2005a, 2005b). One species of eucalypt, *Eucalyptus globulus* has been the focus of much quantitative genetic research to address fundamental ecological and evolutionary questions in eucalypt systems (Jordan *et al.* 2000; O'Reilly-Wapstra *et al.* 2002; Whittock *et al.* 2003; Rapley *et al.* 2004a; Stackpole *et al.* 2011; Wallis *et al.* 2011; Costa e Silva *et al.* 2013; Hamilton *et al.* 2013). While genetic-based recovery from lignotubers has been investigated in felled adult stumps of *E. globulus* (Whittock *et al.* 2003), the genetic basis of plant recovery after catastrophic browsing has not been examined at the vulnerable seedling stage. This is an important life history stage in this species, and in competitive field trial environments, early growth is significantly correlated with probability of later age survival (i.e. size dependent mortality; Chambers *et al.* 1996) and growth (e.g. Stackpole *et al.* 2010). This information will provide a better ecological and evolutionary understanding of eucalypt recovery mechanisms, and how seedling-herbivore interactions can impact community composition and structure (Barton and Hanley 2013). *Eucalyptus globulus* is a globally important plantation species (Dutkowski and Potts 1999) and understanding the impact of severe mammalian browsing damage on plant survival and growth consequence also has applied relevance by enabling prediction of tree productivity after a browsing event, and the implementation of herbivore strategies in managed systems, such as the use of genotypes with improved recovery.

Diversification of morphological traits among populations of *E. globulus* also presents an opportunity to explore recovery strategies based on resource allocation theory. Many plants employ a range of both resistance and recovery mechanisms (Mauricio *et al.* 1997) to cope with multiple herbivore damage across all life stages (Nunez-Farfan

et al. 2007; Fornoni 2011). Despite the lack of support for trade-offs between resistance and recovery mechanisms (Muola *et al.* 2010; Tucker and Avila-Sakar 2010; Oduor *et al.* 2011), we may expect a stronger association between these factors at the vulnerable and resource limited seedling stage (Boege and Marquis 2005; Hanley *et al.* 2007). In the case of *E. globulus*, there are specific population differences that may signal a resource allocation trade-off between growth, storage and defensive chemistry, whereby populations representing the low and high extremes of genetic resistance to marsupial browsing in Tasmania (St Helens and Blue Gum Hill; O'Reilly-Wapstra *et al.* 2002, 2004) have respectively large and small lignotubers (Whittock *et al.* 2003). Theory predicts the existence of trade-offs between defence and growth that could maintain the genetic variation for resistance and recovery observed in plant populations (e.g. Fritz and Simms 1992; Strauss and Agrawal 1999; Agrawal 2011). An important question in the interaction between *E. globulus* and its herbivores is, does this difference in resource allocation impact on tree recovery from browsing damage?

Here I investigated intraspecific variation in seedling resprouting from lignotubers after severe artificial browsing in two *E. globulus* populations, St Helens and Blue Gum Hill. I also report on foliar physicochemical properties after 12 months growing in the field, subsequent lignotuber and stem base diameter up to 20 months, and plant growth and shape up to 32 months. Specifically I asked the following questions:

1. Is there a genetic basis to *E. globulus* seedling lignotuber development and coppice response to catastrophic browsing damage?
2. Are there differences in growth, shape and foliar physicochemical profile with time after browsing and do those differences vary among *E. globulus* populations?

4.3 Methods

4.3.1 Experimental design and assessments

Eucalyptus globulus has been classified into 13 genetically differentiated broad geographical groupings (Dutkowski and Potts 1999). Populations within these groups

are trees growing within 10km of each other (Potts and Jordan 1994), and family is progeny derived from open-pollinated seed collected from a native tree within a population. Sample size was based on previous quantitative genetic studies in this system (e.g. O'Reilly-Wapstra *et al.* 2005a; Wiggins *et al.* 2008) by assessing statistical power and significance of findings. Ten families from two native Tasmanian *E. globulus* populations (i.e. total of 20 families) were selected to represent two geographically and genetically different populations in Tasmania (O'Reilly-Wapstra *et al.* 2002, 2004, 2005). These populations also represent the extremes of browsing resistance. The population from St Helens has relatively low foliar defensive chemistry, and originates from a distinct geographic area in north-eastern Tasmania (latitude 41' 15 S. longitude 148' 19 E) characterised by low rainfall and dry forests. The population Blue Gum Hill is in the wet southern forests of Tasmania (latitude 43' 03 S. longitude 146' 52 E) and has relatively higher foliar chemistry (O'Reilly-Wapstra *et al.* 2004, 2005). Seedlings were used from a previous experiment and assessments began 5 months following a decapitation treatment. They were initially grown in 50 6x7 cell plastic seedling trays filled with slow release fertiliser potting mix (Permium + CRF, low P; Table 4.1).

Table 4.1. Detailed timeline of *E. globulus* recovery trial up to 32 months after decapitation treatment. A single assessment of recovery traits was made on plants in the outdoor nursery at various times after treatment, including mortality, lignotuber size, stem diameter at the lignotuber, height, regrowth shoot length and number of regrowth shoots. At 14 months after treatment, the plants were transferred to a field trial where they were assessed three times. Tree recovery traits assessed at 14 and 20 months after treatment were lignotuber size, stem diameter at the cotyledonary node. Traits assessed at 14, 20 and 32 months were plant height, number of leaders and tree shape.

	Plant age (months)	Time after treatment (months)	Time after field establishment (months)
Seeds sown in glasshouse	0	-	-
Seedlings moved to outdoor nursery	5	-	-
Decapitation treatments applied	6	0	-
Assessment of recovery traits	11	5	-
Trees planted in the field	14	8	0
Mortality assessment	17	11	3
Field assessment of recovery traits 1	20	14	6
Foliage collected for chemical analysis	23	17	9
Field assessment of recovery traits 2	26	20	12
Field assessment of recovery traits 3	42	32	24

Two labelled seedlings chosen from random from each family in each population, were randomly distributed in each tray (40 seedlings per tray). The seedlings were grown for five months in a glasshouse where each tray was allocated a random position and moved monthly to reduce potential environmental effects. After five months trays were transferred to an outdoor nursery for four weeks to allow seedling leaves to harden. At six months of age, the seedlings of each family were subjected to three different treatments:

- Control (C): Uncut seedlings
- Treatment 1 (T1): Stem cut above node 1 (leaving two nodes remaining)
- Treatment 2 (T2): Stem cut above the cotyledonary node 0 (leaving one node remaining; most severe damage)

Treatments were randomly allocated across trays in an unbalanced design, since the seedlings were used from a previous experiment. There were between 76-360 seedlings spread across 8-10 families per population in each treatment (St Helens controls n=313, cut above node 1 n=115, cut above node 0 (cotyledonary node) n=360; Blue Gum Hill: controls n=145; cut above node 1 n=76, cut above node 0 n=274). Following the decapitation treatments, the seedlings resprouted from present lignotubers (node 0 and node 1; Fig. 4.1). Short-term recovery was assessed five months later using the following measures: mortality, lignotuber size at cotyledonary node, stem diameter (taken at the cotyledonary node perpendicular to the lignotubers), height, regrowth shoot length and number of regrowth shoots (Table 4.2). Lignotuber size was calculated by subtracting the stem diameter from the lignotuber diameter and dividing that by the stem diameter at the cotyledonary node perpendicular to the lignotubers (Whittock *et al.* 2003). Stem diameter at the cotyledonary node was also used to determine whether the lignotuber size varied due to stem shrinking or an increase in meristemmatic buds/carbohydrate store. Height was taken from the cotyledonary node 0 to the base of the apical bud of the stem (control plants) or the tallest shoot (treatment plants). Control plants were single stemmed with an average of 16 nodes from the lignotuber (cotyledonary node) to the apical bud.

A field trial was established to assess the longer term recovery response of seedlings to the decapitation treatment. Eight months after treatment, a total of 556 seedlings selected at random were planted in a common garden trial in south-eastern Tasmania, on the Tasman Peninsula (latitude 43˚ 007’ S. longitude 147˚ 055’ E). The northern and eastern sides of the trial site were adjacent to native forest, whilst the remainder was surrounded by early establishing plantation trees. Planted in April (mid-autumn), the trial was arranged in a 10 replicate block design. Each replicate block contained a random arrangement of one seedling for each family by treatment combination (2 populations x 8-10 families x 3 treatments x 10 replicates = 556 seedlings in total). The area (approximately 1 hectare) was enclosed with a net fence to minimise further damage to plants by mammalian herbivores. High seedling mortality (32%) was observed during the three months after trial establishment. Survival and the height, number of leaders and tree shape of survivors was assessed at 14, 20 and 32 months after treatment. Tree canopy width was measured across the widest point of each tree.

Width to height ratio was used to calculate tree shape. The diameter of lignotubers including the stem, and stem diameter at the cotyledonary node perpendicular to the lignotubers was measured 14 and 20 months after treatment. After this time the lignotubers became inaccessible as the stem extended into the soil. The net fence was removed nine months after garden trial establishment, and plants that subsequently received mammal browsing damage were removed from further analysis. No significant insect browsing damage occurred during this time period.

4.3.2 Physicochemical profile

Near infrared spectroscopy (NIRS) was used to characterise the changes in the holistic physicochemical profile (O'Reilly-Wapstra *et al.* 2013a) of juvenile foliage samples harvested from T2, T1 and control plants at 17 months after treatment. Only plants that did not receive further mammal browsing damage in the field were sampled to test for any long-term differences in responses to the treatment (n=206). Three fully expanded young juvenile leaf pairs were picked from three branches of each plant (9 leaves total). Leaves were freeze-dried and later assessed with near infrared spectroscopy (NIRS) as described by Borzak *et al.* (2015a, Chapter 5). Five randomly selected freeze-dried leaves from each sample were scanned with NIR radiation using a 204 stationary, lab-based Bruker MPA FT-NIR spectrometer coupled to a fibre-optic probe instrument. Leaves were scanned both toward the tip and the base, with the midrib areas avoided. The recorded scan from each position comprised the average of four scans on the one spot on the leaf. The resulting ten spectra (2 positions on each of 5 leaves) were then averaged for each tree.

4.3.3 Statistical analysis

The impact of the decapitation treatment on growth traits was investigated using a mixed model analysis (PROC MIXED; SAS Institute Inc. 2009) on data from seedlings at 5 months after decapitation treatment. The fixed effects in the model were population, treatment and all their interaction terms. Random terms were *tray* and *family nested within population* and its interaction with treatment. The survival of seedlings at 5 months after decapitation and in the field 3 months after trial establishment was analysed using a binary model fitted with a logit link function

using PROC GLIMMIX of SAS. The fixed effects in this model were population, treatment and their interaction term. *Replicate*, and *family* nested *within population* together with its two-way interaction with *treatment*, were the random effects in the model. Due to high survival, the 5 month mortality analysis simply tested the main effects of treatment and population; control plants were not included as their mortality was 0%. Data from plants in the field that were assessed multiple times were analysed using a repeated measures mixed model analysis (PROC MIXED of SAS) with seedling being the subject. The fixed effects in this model were population, treatment, time and all their interaction terms. *Replicate*, and *family nested within population* together with its two- and three-way interaction with *time* and *treatment*, were the random effects in the model. Multiple pair-wise comparisons of significant effects at each assessment period were made using the Tukey-Kramer adjustment. Trees that received mammal browsing in the field were omitted from the analysis for that period, and from future assessments. Tree height and lignotuber size were log transformed for the field data analysis.

Treatment responses in the foliage physicochemical profile of sampled leaves were investigated by analysing the reflectance wavelengths from NIR scans. The dataset was transformed using extended multi-scatter correction and reduced to 20 principal components using Principal Components Analysis (The Unscrambler 10.1 of Camo Software 2011; the first two principal components explained 51% and 29% of variation, respectively). Canonical discriminant analysis (PROC DISCRIM of SAS) based on all 20 principal components was used to test and summarise the differences between populations (St Helens and Blue Gum Hill) and treatments (control, decapitation to node 0 and decapitation to node 1). This analysis treated each combination of population and treatment as a separate group. Pairwise comparisons of the mahalanobis distance between groups were calculated to show differences in the holistic physicochemical profile between the two *E. globulus* populations.

4.4 Results

4.4.1 Survival and short-term recovery in the nursery

There was 13% greater survivorship of St Helens relative to Blue Gum Hill 5 months after decapitation ($F_{1,18}$=33.9, P<0.001). No mortality occurred in the controls in either population. Seedlings cut above node 1 (T1) survived 9% better than those cut to node 0 (T2; $F_{1,917}$=12.1, P<0.001). There was close to 100% survivorship of St Helens plants under T1, and the population by treatment interaction term was removed to simplify the model.

For the surviving seedlings, the decapitation treatment had significant short-term impacts on 5 month plant recovery, with induced changes in lignotuber size, stem diameter at the lignotuber, shoot length and shoot number (Table 4.2). Lignotuber size of control plants was greater in St Helens plants (inherently low in foliar chemicals), with values of both populations matching those found by Whittock *et al.* (2003). Lignotuber size increased with treatment severity in both populations. Stem diameter also increased with treatment severity with St Helens once again showing a greater response. The significant interaction term was due to a proportionally greater response to the treatment in Blue Gum Hill plants. The significant population difference in seedling height derived from increased growth in St Helens plants. Shoot length showed a treatment effect, with longer shoots produced by plants with only a single node remaining (T2: most severe treatment). The treatment by population interaction was not significant in shoot number and shoot length, indicating a consistent response of populations to the treatment. St Helens plants produced a greater number of shoots in response to decapitation treatments than those from to Blue Gum Hill. Treatment plants with 2 nodes remaining (T1) produced a greater number of shoots than plants that received the most severe decapitation (T2).

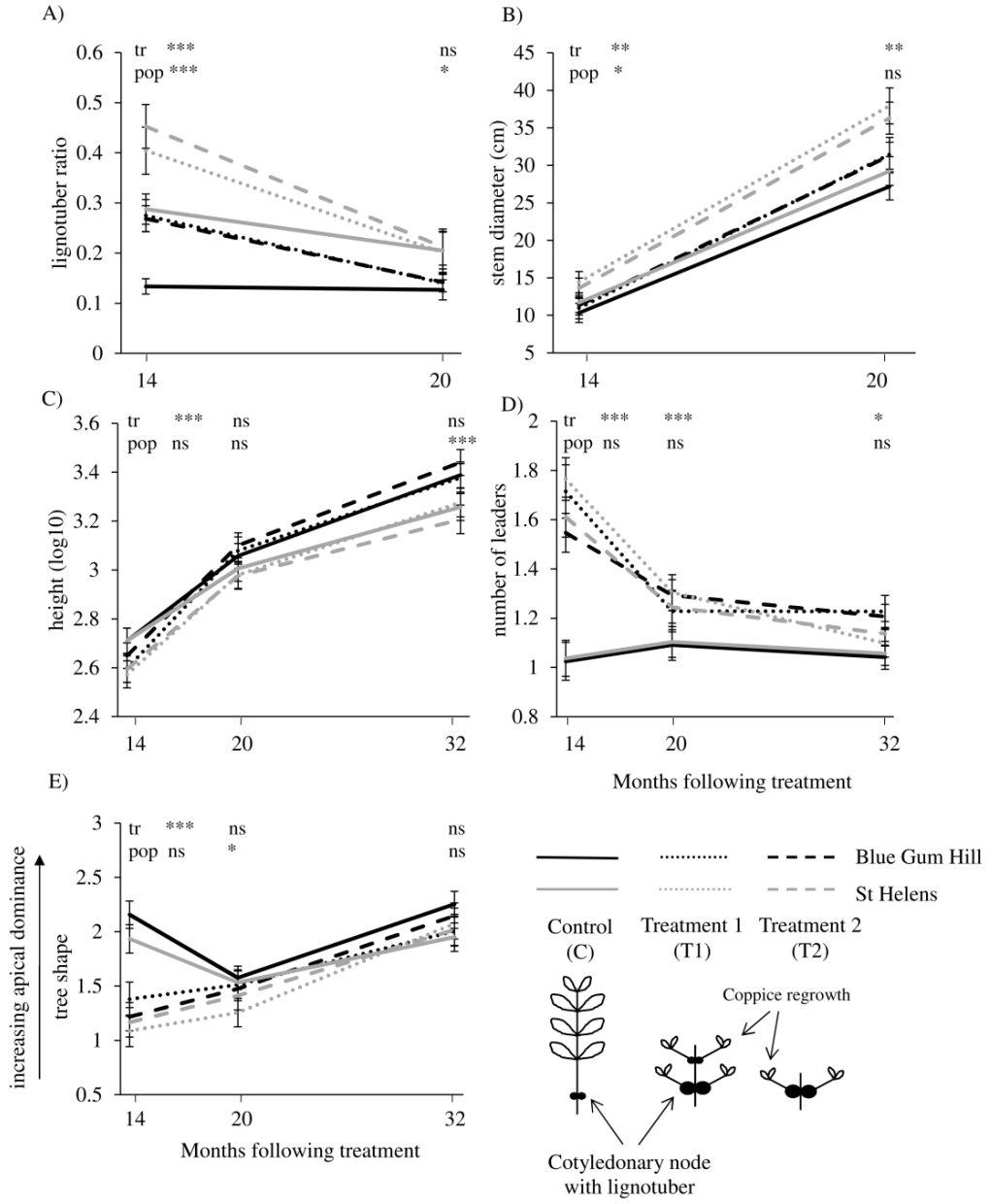

Figure 4.1(A-E). The long-term response of two *E. globulus* populations to decapitation treatments. The means and standard errors are based on family means. Plants were grown in the nursery and were subjected to either decapitation to above the first leaf node (node 1) or above the cotyledonary node (node 0) at six months of age. Plants were left to recover in pots for eight months before being planted in the field (14 months after treatment). Control plants were singled stemmed with approximately 16 nodes. Stem diameter was measured at the cotyledonary node perpendicular to the lignotuber. Tree shape is represented by the height to width ratio. Significant treatment (tr) and population (pop) effects following mixed model analysis for traits at each assessment period are indicated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Mixed model analysis of these results is given in Table 4.3. The population by treatment interaction showed no significant effect for any of the traits. The illustration in the lower right depicts the seedlings at the time of trial establishment, showing the three treatments and the position of lignotubers and sprouts.

Table 4.2. Response means for surviving *E. globulus* seedlings from two populations (St Helens and Blue Gum Hill) at 5 months after decapitation treatment in the nursery. The means and standard errors are based on family means. The treatment was decapitation either above the first seedling leaf node (T1) or above the cotyledonary node (T2). Measurements that were not applicable to the treatment are indicated with n/a. Significance of effects indicated in bold: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

	St Helens			Blue Gum Hill			Population	Treatment	Population *Treatment
	T1	T2	C	T1	T2	C			
N	313	115	360	145	76	274			
Lignotuber size	0.72 ± 0.05	0.84 ± 0.04	0.35 ± 0.04	0.46 ± 0.05	0.57 ± 0.04	0.18 ± 0.05	**16.3*** **	**139.9*** **	**3.8***
Stem diameter	3.71 ± 0.12	3.96 ± 0.11	3.50 ± 0.13	3.55 ± 0.14	3.79 ± 0.11	2.92 ± 0.14	**14.4*** **	**11.3****	**8.6*** **
Regrowth shoot length	87.3 ± 4.1	96.6 ± 3.3	n/a	81.7 ± 4.7	99.0 ± 3.4	n/a	0.3	**21.1*** **	2.1
Regrowth shoot number	4.57 ± 0.21	3.76 ± 0.17	n/a	3.34 ± 0.23	2.82 ± 0.18	n/a	**22.2*** **	**24.6*** **	1.3
Height	n/a	n/a	386.3 ± 8.7	n/a	n/a	321.6 ± 12.9	**8.9*** **	n/a	n/a

4.4.2 Survival and long-term recovery in the field

Three months after planting in the field, a high proportion of plants (32%) failed to establish. Plant mortality was likely due to drought stress and did not differ between the two populations ($F_{1,17}$=3.9, P=0.06). There was a significant treatment effect ($F_{2,36}$=5.8, P=0.006) with 16% and 30% greater survivorship in the uncut control plants relative to the decapitated treatment plants T1 and T2, respectively. There was no treatment x population effect. Thereafter, there was no further mortality in the trial. The total number of surviving plants was 377, with 23%, 34% and 41% loss of control, T1 and T2 plants, respectively. Per treatment the number of live plants remaining was 154, 131 and 92 in controls, T1 and T2 respectively.

Among these surviving plants there were significant treatment effects in many recovery traits (Table 4.3, Fig. 4.1). The surviving seedlings in the field showed treatment effects on lignotuber size, stem diameter at the lignotuber, tree height, number of leaders and tree shape over multiple assessments (Fig. 4.1A-E). Over time the treatment effect on lignotuber size, height and shape disappeared. There was a clear population effect on lignotuber size and stem diameter at the lignotuber. At 14 months after decapitation (six months after planting) these were greater in St Helens than Blue Gum Hill (Fig. 4.1A and 4.1B). At 20 months, the treatment effect on lignotubers disappeared but the population difference remained. Conversely, stem diameter at the cotyledonary node (perpendicular to cotyledons) at 20 months, showed a treatment effect but no population effect. The increase in stem diameter at the cotyledonary node resulted from the decline in lignotuber size from 14 to 20 months. Decapitation reduced plant height (Fig. 4.1C) at 14 months, but by 20 months the effect of treatment had disappeared, demonstrating the regrowth capacity of this species. However, the population effect on height remained, with a tendency for St Helens seedlings to be shorter than those from Blue Gum Hill. At 14 months, the number of stem leaders was higher in the decapitation treatments (Fig. 4.1D). Although leader numbers decreased significantly over time, the treatment effect remains at 32 months.

Table 4.3. Results of the repeated measures analysis of variation in recovery traits of *E. globulus* for population, treatment, time and all their two- and three-way interaction levels. Plants were subjected to either decapitation to above the first leaf node (T2) or above the cotyledonary node (T1, see Fig. 4.1). Lignotuber size and stem diameter were assessed at 14, 20 months after decapitation treatment (time factor). Height, shape and number of leaders were assessed at 14, 20 and 32 months after decapitation treatment. Ndf = numerator degrees of freedom for fixed effects. Ddf = denominator degrees of freedom associated with the random error terms used to test the fixed effects. Significance of effects indicated in bold: **, P < 0.01; ***, P < 0.001.

		Lignotuber size	Stem diameter		Height	Shape	Number of leaders
	Ndf, Ddf	F	F	DF	F	F	F
population	1,18	**16.7*** **	**13.2*** **	1,18	**15.9*** **	4.2	0.0
treatment	2,35	**9.2*** **	**8.2*** **	2,35	1.4	**21.6*** **	**31.6*** **
time	1,51	**95.3** **	**1254.0*** **	2,104-106	**1118.1*** **	**108.4*** **	**41.9*** **
population*treatment	2,35	0.8	1.0	2,35	1.4	0.4	0.1
population*time	1,51	2.9	4.0	2,104-106	**10.4*** **	0.3	1.5
treatment*time	2,51	**11.5*** **	**8.7*** **	2,104-106	**8.8*** **	**15.4*** **	**13.7*** **
population*treatment*time	2,51	0.1	0.6	2,104-106	1.9	2.2	0.8

In terms of tree shape (Fig. 4.1E), St Helens plants exhibited less apical dominance than those from Blue Gum Hill, but this was not statistically significant. Treatment affected shape initially, with increased lateral growth regardless of population. Whilst the control trees showed reduced apical dominance at 20 months, the shape of treatment trees improved at this time, with increased height and reduced lateral growth. At 32 months, apical dominance continued to increase in the treatment plants and was regained in the control trees. There was no significant difference in the way the two populations responded to decapitation in terms of number of leaders or tree shape.

4.4.3 Physicochemical profile

Discriminant analysis based on the spectral physicochemical profile of plants 17 months after treatment clearly differed between populations in the space defined by the first two canonical variates (Fig. 4.2). Canonical variate 1 described the holistic physicochemical differences between populations and explained 83% of variation between the groups and was highly significant ($P<0.001$). Canonical variate 2 showed no significant difference among the treatments (population x treatment), explaining only 6.7% of the variation between groups. Pair-wise comparisons of Mahalanobis distance between groups revealed highly significant differences in the holistic physicochemical profile of the two *E. globulus* populations in all cases. By contrast, no significant difference was evident among the treatments of each population.

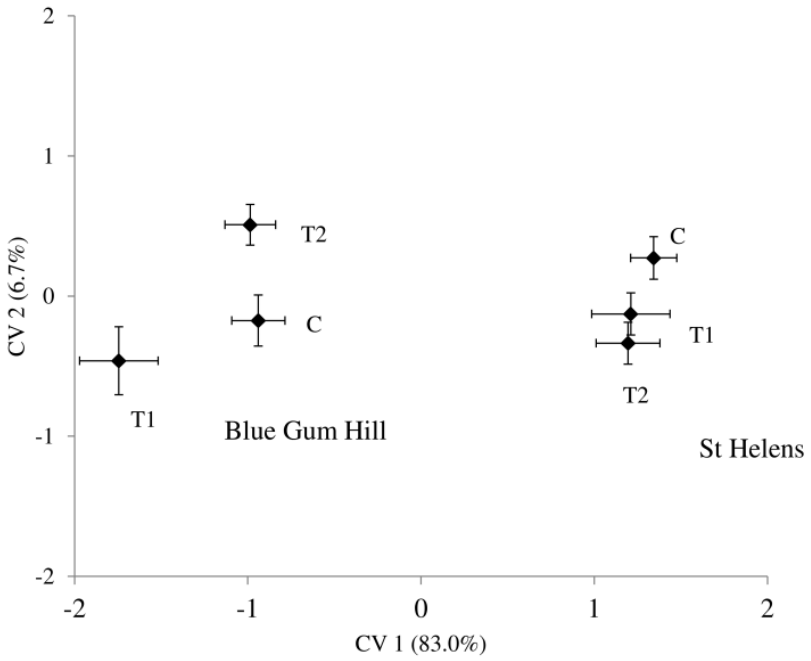

Figure 4.2. The discriminant analysis summarises the physicochemical properties as determined by near infrared spectroscopy, of two *E. globulus* populations (St Helens, n=103; and Blue Gum Hill, n=103) where the uncut controls (C) were compared to the regrowth of plants subject to two severe decapitation treatments (T2 and T1; see Fig. 4.1). Foliage was sampled 17 months after decapitation treatment. Canonical variate (CV) 1 indicates genetic stability with clear groupings of treatment from the same population. Variation between treatments on CV 2 were not statistically significant (P=0.95), whereas variation on CV 1 was highly significant (P<0.001). Pair-wise comparison of Mahalanobis distances between groups showed no difference between treatments within each population, but highly significant differences between populations within treatments.

4.5 Discussion

An important recovery strategy of *E. globulus* is to produce coppice arising from lignotubers (Bond and Midgley 2003; Vesk and Westoby 2004), however differences in the recovery response and long-term consequence of seedling decapitation between populations are largely unknown. There were two important findings from the present experiments. The first was the significant difference in survivorship among *E. globulus* populations with inherently large and small lignotubers (St Helens and Blue Gum Hill respectively; Whittock *et al.* 2003). Increased survivorship, i.e. sprouting ability, was associated with better developed lignotubers. The second was the overwhelming stability among *E. globulus* populations in the short- and long-term

growth recovery responses in the surviving seedlings after decapitation. Despite the conservative nature of recovery traits, the populations demonstrated constitutive differences which, in the long-term, were overridden by treatment effects.

Greater seedling survivorship of the *E. globulus* population St Helens than blue gum hill population is consistent with its larger lignotubers and stem diameter (at the cotyledonary node). This evidence strongly supports the association between lignotuber size and stem diameter and increased tree sprouting ability (Whittock *et al.* 2003). In the event of severe biomass loss, eucalypts rely on stored carbohydrate reserves to produce coppice from lignotubers (Bond and Midgley 2001; Noble 2001; Walters *et al.* 2005a; Clarke *et al.* 2013). Genetic variation in traits that determine the success of seedling resprouting such as survival, as seen here, suggests that divergent selection has occurred among *E. globulus* populations with St Helens able to better potentially tolerate catastrophic damage through greater resource storage. Increased survivorship after severe loss of biomass, i.e. the ability to resprout from stored reserves, may have multiple origins. It may be an adaptive response to catastrophic damage from a number of biotic (e.g herbivory) and/or abiotic stresses (e.g. drought, fire and wind), and such interactions may vary with plant genotype and plant age (Cruz and Moreno 2001; Del Tredici 2001; Aparicio *et al.* 2015). When considering the selective forces that drive resource allocation trade-offs, there is an obvious pattern in *E. globulus* whereby populations that express inherently lower chemical defences are more prone to mammalian herbivory (O'Reilly-Wapstra *et al.* 2005a) and may perform better in response to damage. This leads me to hypothesise that increased allocation to storage reserves is an alternative mechanism to foliar defences for eucalypt populations to survive browsing damage. This may often involve resource allocation trade-offs between growth, storage and defence, that are difficult to untangle (Agrawal *et al.* 2010).

Recovery traits of the surviving seedlings not only demonstrated genetic stability in constitutive differences, but also in the way they changed in response to the decapitation treatments. The decapitation treatment elicited short-term plastic responses, but after 20 months of growth in the field, the surviving plants showed no lasting treatment effects on important growth traits, with population differences

overriding treatment effects. This demonstrates that surviving *E. globulus* seedlings were able to fully recover in this time-frame, from a once-off severe decapitation event. Stable responses were also detected in foliar physicochemical properties (at 17 months) with no long-term change in defensive chemistry after decapitation. A similar lack of long-term response in physicochemical profile after varying levels of browsing damage is reported in Chapter 5 of this thesis (Borzak *et al.* 2015a, Chapter 5). To date there has been little evidence of foliar chemical induction in *Eucalyptus*, with the only reported cases exhibiting rapid rather than delayed responses (Rapley *et al.* 2008). The observed genetic stability in the numerous recovery responses among the two *E. globulus* populations in this study suggests that despite intraspecific variation in the allocation of stored resources to lignotubers (and stem base), the strength of selection on recovery traits is not strong. This suggests that recovery mechanisms that are employed to mitigate the negative effects of severe defoliation among *E. globulus* populations are stable. My findings add to the growing body of work showing stable responses to environment in this species (McKiernan *et al.* 2012; O'Reilly-Wapstra *et al.* 2013a; O'Reilly-Wapstra *et al.* 2013b), including evidence in Chapter 3 of this thesis. Genetic stability of traits in *E. globulus* has important applied implications for reforestation projects and commercial plantations; for example, the lack of variation in recovery traits among populations will give growers confidence that artificially selected genotypes for improved herbivore resistance (Miller *et al.* 2009), will demonstrate the same recovery responses if browsing damage does occur.

Growth traits and physicochemical profiles of the surviving plants exhibited constitutive differences among *E. globulus* populations in both the short- and long-term assessments. Coppice production in response to decapitation resulted in a higher incidence of double leaders in the short-term, a response consistent with plant-mammal studies that show the increased production of multiple stems following severe marsupial browsing damage (Close *et al.* 2010; Borzak *et al.* 2015a, chapter 5). In the short-term, control plants exhibited a loss in apical dominance with seedlings producing greater lateral growth. This was likely to be a result of establishment stress as seedlings acclimatise to the site conditions (Close *et al.* 2003) or related to the effects of container size on root architecture (Close *et al.* 2006). Despite these patterns, early shoot attrition reduced the long-term effect on tree shape in both

treatment and control plants. Attrition of eucalypt coppice shoots from lignotubers has been previously attributed to competition, apical dominance, or incomplete development of the bud vascular trace (Graham *et al.* 1998; Wildy and Pate 2002). At 32 months, growth of St Helens plants showed a significant decrease in above-ground growth, which is consistent with quantitative genetic studies showing north-eastern Tasmanian *E. globulus* populations tend to be slower growing than southern populations (Stackpole *et al.* 2010). Although the possibility that the poor growth performance of St Helens is a result of mal-adaption to the more southern field site cannot be dismissed (Volker and Orme 1988; Costa e Silva *et al.* 2014), this pattern is consistent with the hypothesis that allocation of resources to active reserve formation may carry a cost trade-off against growth, maintenance and reproduction (Chapin *et al.* 1990; Bond and Midgley 2001; Poorter *et al.* 2012; Clarke *et al.* 2013; Nzunda *et al.* 2014). Delays in reproductive traits are characteristic of *E. globulus* populations in north-eastern Tasmania and include delayed vegetative phase change (Jordan *et al.* 2000) and flowering precocity (Chambers *et al.* 1997) in the population of St Helens. Trade-offs in growth and reserve storage are likely to reflect the growth strategy of eucalypts in wet/dry environments (Ladiges 1974), where populations from southern Tasmania (e.g. Blue Gum Hill) are driven by competition and therefore invest more resources in above ground growth. In contrast, populations such as St Helens from north-eastern Tasmania are adapted to drier environments, with traits such as thicker bark to cope with drought stress (Dutkowski and Potts 2012).

Differential patterns in resource allocation among populations are likely to have additional consequences to the plant in the event of multiple defoliation events, for example negative impacts on fitness from delayed phase change and flowering (Bulinski and McArthur 1999; O'Reilly-Wapstra *et al.* 2012; Borzak *et al.* 2015a). Under repeated browsing conditions, there is also a potential for populations with higher levels of chemical defence to fail to maintain shoot regrowth sooner, than those with lower chemical defences and better regeneration mechanisms (Reudler *et al.* 2013).

In conclusion, the observed differences in lignotuber size and stem diameter have fitness consequences on seedling survival following decapitation, and are therefore

key traits during juvenile stage growth. The evidence shown in this study suggests that increased resource allocation to these structures is an adaptive mechanism that enhances seedling survival after severe loss of biomass. This may be a response to severe browsing by herbivores, or other disturbances that cause decapitation, such as drought and wind, and is consistent across *E. globulus* life stages (Whittock *et al.* 2003). The plants that survived seedling decapitation showed no longer lasting impacts across a 32 month period, from severe damage on important growth traits, and demonstrated genetic stability in the recovery strategy among *E. globulus* populations. These results have important applied implications for managed systems, such as forest restoration and commercial plantation. For example, growers artificially selecting genotypes for traits such as improved solid-wood value (Callister *et al.* 2011) or increased defensive chemistry to reduce herbivore damage (Miller *et al.* 2009) maybe confident of the same recovery response to any disturbance.

4.6 Acknowledgments

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Chapter 5:
Direct and indirect effects of marsupial browsing on a foundation tree species

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5.1 Abstract

Plant-mediated indirect effects can be important ecological drivers in plant communities, especially in systems where extended genetic effects of foundation species can shape communities and influence ecosystem dynamics. Here we investigate the direct and indirect effects of uncontrolled browsing by marsupial herbivores including the common brushtail possum (*Trichosurus vulpecula*), Bennetts wallaby (*Macropus rufogriseus*) and the red-bellied pademelon *(Thylogale billardierii)*, in a *Eucalyptus* system known to have extended community and ecosystem genetic effects. In a common garden trial containing 525 full-sib families from an incomplete diallel crossing program located in north-eastern Tasmania, Australia, we assessed the genetic basis to herbivore preferences, the impact of a single and repeated marsupial browsing event on tree fitness and morphological traits and the associated indirect plant-mediated effects on a subsequent herbivore, autumn gum moth (*Mnesampela privata)*. Marsupial browsing was not influenced by plant genetics, but spatial components instead affected the pattern of damage across the trial. Marsupial browsing had significant impacts on tree survival and morphological and developmental characteristics, resulting in reductions in survival, height and basal area, an increased proportion of multiple stems, delays in phase change from juvenile to adult foliage and delays in flowering. Minimal fitness impacts were observed in

response to a once-off browsing event, but effects were exacerbated when trees suffered repeated browsing. We demonstrate clear plant-mediated indirect effects of marsupial browsing on subsequent tree use by an invertebrate herbivore, through induced changes in plant morphology. Such indirect effects have the potential to influence biotic community structure on a foundation species host-plant, and the evolutionary interactions that occur between organisms and the host-plant themselves.

5.2 Introduction

Plants are consumed by a variety of different herbivores across all life stages, from seedling, to juvenile to adult. Multiple species of vertebrate and invertebrate herbivores, covering all feeding guilds, can feed on the plant at the same time across temporal scales (Strauss and Irwin 2004). Herbivores may interact with each other either directly or indirectly and their interactions may be genetically (e.g. genetic covariance in preferences) or ecologically (e.g. competition) based (Wise 2009). It is therefore becomingly increasingly clear that a multi-species approach is required to fully understand the ecological and evolutionary dynamics between plants and their herbivores (Utsumi 2011, 2013; Walsh 2013).

The direct effect of herbivores on plants can lead to a range of phenotypic changes (Ohgushi 2005). Thus, through browsing, one herbivore may indirectly affect other herbivores and even interactions at other trophic levels (Werner and Peacor 2003). For example, marsupial herbivory in woody plant systems can impact the subsequent community positively through changes in tree architecture as a result of vigorous regrowth (Bailey and Whitham 2006). In contrast, negative associations can occur, for example in response to marsupial herbivory through reduced plant quality (Lind *et al.* 2012) or induced hardening of leaves (Shimazaki and Miyashita 2002). Such induced trait changes are important ecological and evolutionary drivers in many systems as they may alter the abundance, composition and viability of subsequent herbivore species or pathogen communities on plant species (Ohgushi 2005). This in turn may have significant consequences for plant community composition and ecosystem dynamics (Utsumi 2011), highlighting the importance of such studies on foundation tree species. Intraspecific genetic variation in the host plant may have further

consequences on the community-level biodiversity, with genetic-based differences affecting plant-herbivore interactions (Whitham *et al.* 2003).

Eucalypts are a dominant genus throughout Australian forests, with intraspecific genetic variation that can have community and ecological consequences (Barbour *et al.* 2009a). Eucalypt foliage may be infected with a number of fungal pathogens (Keane *et al.* 2000), and consumed by a number of invertebrate (Rapley *et al.* 2004a, c; Steinbauer and Matsuki 2004) and vertebrate herbivores (Bulinski and McArthur 1999). To date, studies within eucalypt-herbivore systems have focused on understanding inter- and intra-specific feeding preferences (O'Reilly-Wapstra *et al.* 2002; Wiggins *et al.* 2006a) and the genetic basis of herbivore preferences (Jones *et al.* 2002; O'Reilly-Wapstra *et al.* 2004; Rapley *et al.* 2004b). Also well-studied are the impacts of herbivore damage on plant traits, such as eucalypt survival (Bulinski and McArthur 1999), growth (Pinkard *et al.* 2006b), plant shape (Bulinski and McArthur 1999; Close *et al.* 2010) and reproductive fitness (O'Reilly-Wapstra *et al.* 2012). Little research, however, has focused on how herbivores in eucalypt systems interact to directly affect each other's feeding preferences (e.g. Mar and McArthur 2005) and even less has addressed the indirect plant-mediated effects of herbivores. Such indirect interactions are important to consider given the vast array of herbivores and pathogens that consume eucalypts, potentially resulting in significant herbivore-induced effects on tree phenotype. Studies addressing this question will provide a more complete picture of the ecological and evolutionary implications of herbivore-plant interactions in eucalypt systems.

One avenue where eucalypts may affect subsequent biota after browsing is in their propensity to regrow. Following defoliation, eucalypts can recover by producing coppice from dormant buds (Vesk and Westoby 2004). The new coppice foliage potentially has different phenotypic characteristics to previous foliage (O'Reilly-Wapstra *et al.* 2007) and, consequently has the capacity to indirectly affect the subsequent colonising herbivore or pathogen community (Steinbauer *et al.* 1998). These herbivore-induced responses may be variable and depend on a multitude of genetic and environmental factors, including damage frequency (Wills *et al.* 2004),

timing (Pinkard and Beadle 2000), severity (Pinkard 2003) and pattern of defoliation (Pinkard *et al*. 2006a).

Here we investigate the direct and indirect effects of uncontrolled marsupial browsing in a planted forest of the foundation tree species *Eucalyptus globulus,* where seedlings were attacked by marsupial herbivores and later by an invertebrate herbivore, autumn gum moth. Specifically we asked the following questions:

1. Is there a genetic basis to variation in herbivore damage on different *E. globulus* seedlings?
2. What are the direct effects of marsupial browsing on *E. globulus* seedling traits and fitness?
3. What are the indirect effects of marsupial browsing on later herbivory by autumn gum moth (AGM)?
4. What are the plant mediated traits influencing these indirect effects?

5.3 Materials and Methods

5.3.1 Plant pedigree and field trial design

Over 8000 *Eucalyptus globulus* trees of known genetic stock were planted in June 2007 in a common environment field trial in north-eastern Tasmania, Australia (latitude 40˚ 96’ S. longitude 147˚ 98’ E). *Eucalyptus globulus* is a dominant forest species with a natural distribution in south-eastern Australia. This species has been classified into 13 genetically differentiated, broad geographical groupings called races (Dutkowski and Potts 1999). Approximately half of the trees in this trial (n=4434) consisted of 525 full-sib families from an incomplete diallel which included reciprocals (Costa e Silva *et al.* 2013). This diallel involved both intra- and inter-race crossing amongst ten parents from each of three *E. globulus* races - Furneaux (F), Strzelecki (S) and Otways (O). The parents were grafted selections made by the Southern Tree Breeding Association (STBA) from families derived from native stand open-pollinated seed collections that had been grown in base population trails of *E. globulus* in Australia. All of the seed parents and most of the pollen parents used were grafts that had been established in a seed production facility in Tasmania by

SeedEnergy Pty Ltd (Potts *et al.* 2008). The number of full-sib families in the diallel trial studied was as follows: FxF (82), FxS (83), FxO (69), SxF (54), SxS (72), SxO (47), OxF (38), OxS (50), OxO (30). The races used to create the diallel exhibited above average resistance to marsupial browsing (O'Reilly-Wapstra *et al.* 2002). The remaining families in the trial were full-sib families that were part of the routine breeding population of the STBA. Although the majority of the assessments for this study were scored on the entire 8000 tree trial, only the trees within the diallel are analysed in the present study.

The trial was planted on former agricultural land, with trees planted in a rectangular array with a spacing of 2 m within rows (cultivated rip-lines) and 5 m between rows. The trial was planted as a randomised resolvable row-column block design with 15 replicates (Williams *et al.* 2002), each containing 576 trees (Fig. 5.1A). Each family was allocated a single-tree plot within each replicate and seedlings of other families randomly allocated to positions when there were insufficient seedlings of a required family.

5.3.2 Direct impacts of marsupial browsing within fenced and unfenced areas

Full diallel trial

Within the first month after planting, the trial was browsed by free ranging marsupial herbivores. At this site the common marsupial herbivores that could browse the seedlings were the common brushtail possum (*Trichosurus vulpecula;* body mass 4.0 ± 0.5kg), Bennetts wallaby (*Macropus rufogriseus*; body mass 20.0 ± 7.6kg) and the red-bellied pademelon (Thylogale billardierii; body mass 5.5 ± 1.4kg; Mar and McArthur 2005). As we could not separate damage by the three different herbivores on the plants we grouped the damage as collective ‘marsupial damage’. To capture the extent of marsupial damage at this critical developmental stage, foliage remaining on the plant after browsing was scored twice across all 8000 seedlings (moving down rows), in August and then again in September 2007. A seven-point visual estimate was used to record the proportion of foliage browsed: 0= no defoliation, 1=1-5%, 2= 6-25%, 3=26-50%, 4=51-75%, 5=76-95% 6=96-100%.

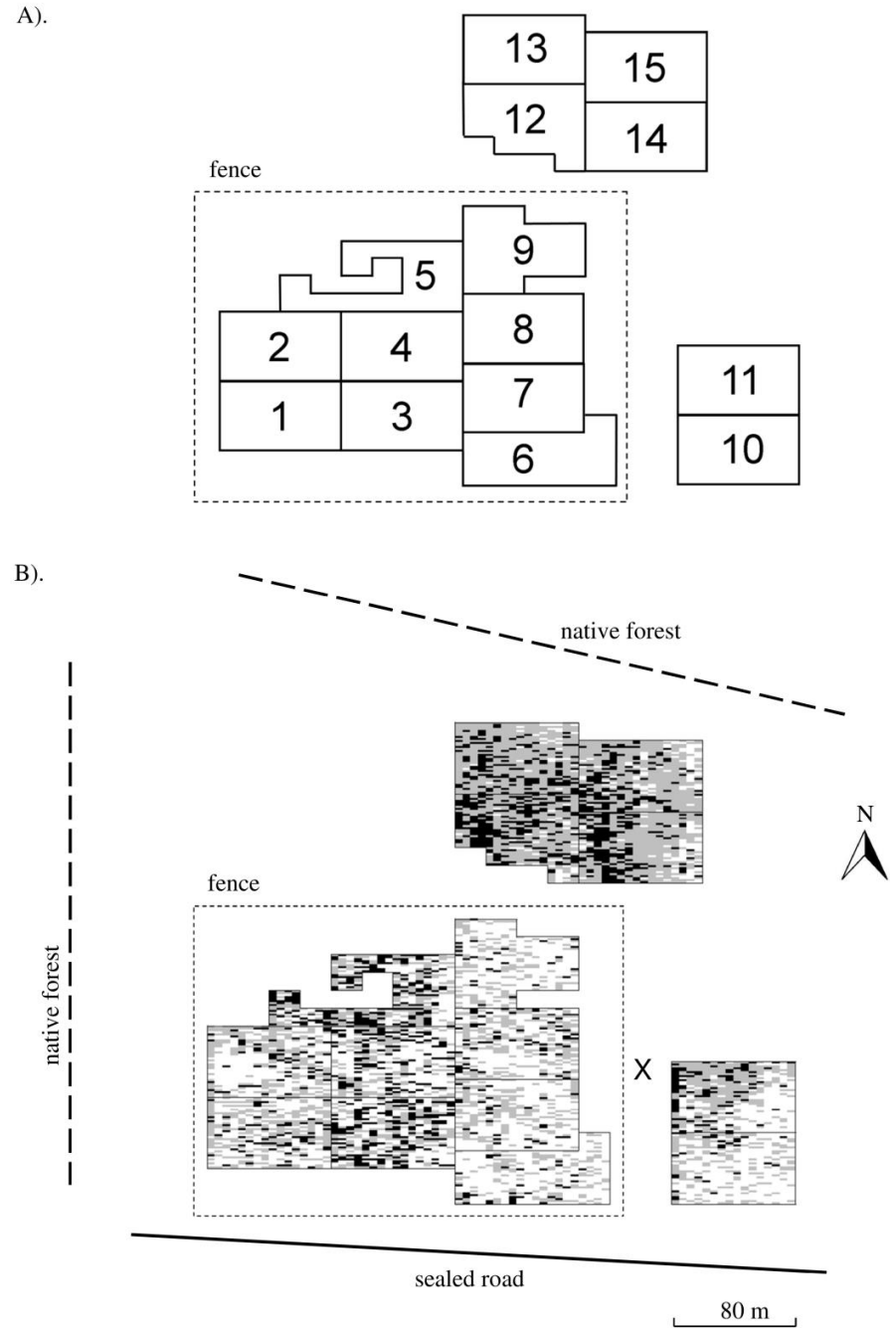

Figure 5.1. Experimental design of trial (A) and average field browsing damage (B). Large numbers indicate 15 replicate blocks and each containing 576 trees. The subset of trees selected for the detailed assessment were from an area of low browsing level (replicates 6-9) and an area of high browsing level (replicates 1-4). Each tree is represented with a coloured square; ☐ 0% browsed; ▒ 15-62.5% browsed; ■ >62.5% foliage browsed. The fine dotted line represents the area of the trial that was fenced and field browsing was scored before the fence was erected. Proximity of the field trial to sealed road and to native forest where marsupials take refuge is shown on the map. X indicates the position of a large pile of logs and stumps that also provided marsupial refuge.

Minimal regrowth had occurred at the time of browsing assessment and browse damage on seedlings was easily recognisable. Following the second assessment of marsupial browsing damage at year 0, a large section of the trial was fenced (9 replicates, n=5184; Fig. 5.1A) to prevent further marsupial damage on the establishing seedlings. This fence was removed in September 2008, one year and 4 months after planting, by which stage the plants had escaped their most susceptible growth period of marsupial browsing. This allowed us to assess the direct impacts of a single browse event and compare it to the response of trees outside the fence that were not protected from further and repetitive browsing damage. This was done by analysing the trees from the diallel within the fenced (n=2658) and unfenced areas (n=1776).

Due to the longevity of *E. globulus*, measures of fitness surrogates were used to assess the effect of browsing damage on survival and reproductive output (O'Reilly-Wapstra *et al.* 2012) each year up to four years post browsing damage (Table 5.1). Traits assessed across the entire trial were tree mortality, height, basal area of surviving plants (including multiple stems), phase change from juvenile to adult foliage and flowering. Stem straightness and the proportion of multiple stems are important traits in solid wood production (Close *et al.* 2010; Callister *et al.* 2011) but were primarily included in this study to represent tree architecture or shape which takes lateral growth into account and can have impacts on subsequent invertebrate communities (Bailey and Whitham 2006). Trees were in juvenile stage for the first three trait assessments, and at 1.7 years after trial establishment, trees exhibited vegetative phase change from juvenile to adult foliage (Jordan *et al.* 1999). Stem straightness was subjectively assessed using a 6-point scale (Blackburn *et al.* 2013), with 1 exhibiting the straightest stems, and 6 bearing the most kinks and curves.

Table 5.1. Summary of trial assessments up to four years post browsing. Trial was planted in June 2007 and 9 replicates were fenced in July 2007. Fitness surrogate traits assessed are mortality, tree height, basal area, vegetative phase change and flower buds. Presence of adult foliage indicates phase change from juvenile to adult.

Assessment time after browsing	**Year**	**Month**	**Traits scored**
-	2007	June	Trial establishment
-	2007	Aug/Sept	Marsupial browsing scored
7 months	2008	April	210 tree subset assessment in fenced area
9 months	2008	June	Autumn gum moth damage
1 year	2008	Sept	Mortality, height, multiple stems
1.7 years	2009	June	Mortality, basal area, adult foliage
3 years	2010	Sept	Mortality, flower buds
4 years	2011	Aug	Mortality, basal area, multiple stems, stem straightness

Fenced subset

A subset of the diallel within the fenced part of the trial and within the diallel was chosen for a detailed assessment (n=210) of tree morphology and foliar physicochemical phenotype to test for possible induced effects from a single browsing event. Plants represented only Strzelecki (34 families) and Furneaux races (71 families). Data on the Otway trees were excluded due to insufficient sample size in the high damage category. Two trees were selected from each family, with one of the family pair received no browsing (0%) and was located in an area of low-level browsing (across 4 replicates). The other plant in the family pair was in an adjacent area which had been subject to a high-level of browsing (another 4 replicates) where seedlings received varying severities of browse damage. In the low-level area all families were represented by unbrowsed plants whereas in the high-browsed area some families were represented by a plant with high browsing (>62.5% foliage browsed), others by plants with intermediate browsing (15 - 62.5%), and others also represented by plants with no browsing (0%). Thus as the same families were sampled in both areas, but in one area they had different levels of browsing and were all unbrowsed in the other area, any significant interaction term between the family browse damage class (0%, 15-62.5% and >62.5% based on the score from the high-level browsing area) and area (low- and high-levels of browsing) would indicate a direct trait/organism response to previous browsing In April 2008, 7 months post

browsing, these 210 trees were scored for canopy height and width, basal area, and number of stems. Tree shape was calculated as height/width.

To test for possible induced chemical effects of marsupial browsing, foliage samples were also taken during the sampling 7 month post browsing. This is particularly pertinent given that a number of *E. globulus* foliar chemical characteristics have been previously linked to AGM oviposition, performance and larval survival (Jones *et al.* 2002; Rapley *et al.* 2004a, d; Steinbauer and Matsuki 2004; Östrand *et al.* 2008). Three young leaf pairs were picked from three branches (9 leaves total). Leaves were freeze-dried and later assessed with near-infrared reflectance spectroscopy (NIRS) to assess changes in the holistic physicochemical profile (O'Reilly-Wapstra *et al.* 2013a) post browsing, i.e. evidence of induced effects. Five randomly selected leaves from each sample were scanned with NIR radiation using a 204 stationary, lab-based Bruker MPA FT-NIR spectrometer coupled to a fibre-optic probe instrument. Leaves were scanned at two positions, using the average of four scans per position, and avoiding the midrib and damaged areas. The recorded scan from each position comprised the average of four scans on the one spot on the leaf. The resulting ten spectra (2 positions on each of 5 leaves) were then averaged for each tree.

5.3.3 Indirect effects of marsupial browsing on subsequent herbivores

Eucalyptus globulus is browsed by a variety of invertebrate herbivores including the autumn gum moth (AGM; *Mnesampela privata*, Guenée; Lepidoptera: Geometridae, Ennominae). Autumn gum moth are endemic to south and south-eastern Australian forests. Female moths prefer to oviposit on juvenile foliage of *E. globulus (Steinbauer 2002)* possibly because of increased larval performance on juvenile leaves (de Little *et al.* 2008; Östrand *et al.* 2008). After hatching, the larvae develop through five instars before pupation, skeletonising the leaf surface until the third instar stage after which they feed on all leaf material (Elliott and Bashford 1978). In the current trial, damage by AGM appeared 9 months (0.7 years) after the browsing event (June 2008) with the majority of damage in the mid-low section of the canopy, by the older larval stages ($3^{rd}$to 5^{th} instar stages) where the leaf, excluding the midrib, was eaten. Damage by AGM was scored from 0 to 3 (0=no defoliation; 1=<10%; 2=10%-49%;

3=>50%), on the basis of the highest level of foliage loss observed on a single branch. Trees were scored below breast height, on the downwind east-facing side, where there was increased presence of AGM larvae. For analysis, AGM damage was converted to a presence/absence score.

5.3.4 Statistical analysis

5.3.4.1 Full diallel trial

The genetic and non-genetic components of variation in herbivore (marsupial and AGM) damage and first year tree mortality and height were estimated by analysing the diallel across the entire trial at one year post trial establishment (n=4434). The marsupial browsing scores was converted to percentage defoliation using the mid-score for each browse category (e.g. browse score 3 = 37.5%) and analysis undertaken on the average of the August and September assessments for each plant. Tree basal was normalised using a square-root transformation. Details of the models used to test for tree genetic and spatial effects on the response traits are given in Supplementary Material Appendix 1. To test for the direct and indirect effect of browsing itself, browsing was treated as an explanatory factor rather than as a response variable. The average defoliation percentage scores were reduced to five browsing damage categories (0%, 15%, 37.5%, 62.5% and 87.5% defoliation) by combining the two highest and lowest defoliation levels. This aggregation was done due to low sample sizes at the lower and higher end of the browsing scale. This five-level browsing class was then used as a factor in models fitted to the data from the full diallel trial as well as the fenced subset.

The diallel plants from the full trial (n=4434) were used in analyses to investigate both the direct impact of a marsupial browsing event on plant traits as well as the indirect effect on subsequent colonising organisms. The differential effect of marsupial browsing on trees within the fenced and unfenced areas was tested by fitting the following linear mixed model to the tree data using the program ASReml (version 3) with a pedigree file used to estimate the additive relationship matrix (Gilmour *et al.* 2009).

The model fitted was:

$$Y = \mu + \text{Fenced} + \text{Browse score class} + \text{Race GCA} + \text{Browse score class.Race GCA} + \text{Fenced.Browse score class} + \text{Fenced.Race GCA} + \text{Fenced.Browse score class.Race GCA} + \textit{Rep(fenced)} + \textit{Row} + \textit{Col} + \textit{Additive} + \varepsilon$$

where Y is the observation, μ is the trait mean, Browse score class is the average browsing damage category (5 levels), Race GCA is the fixed race general combining ability effect that pools the race effects across males and females, Race SCA is the fixed race specific combining ability effect. The two and three way interactions involving Browse score class, Race GCA and Fenced were also fitted and random terms *Rep(fenced), Row, Col , Additive and* ε are as described above. For presence/absence variables a binary model with a logit link function was used. The spatial and within crosstype genetic terms were excluded from the mixed model and were incorporated in the error term because their inclusion did not allow model convergence. Height at year one was also included as a covariate, together with the interaction between height and fence, to investigate if herbivore-induced variation in growth contributes to variation in the presence of AGM damage. For this analysis, all plants with missing height data were removed (leaving a total n=4410).

In both the fenced and unfenced area, the relationship of insect herbivory by AGM and plant traits (height and the proportion of multiple stems) was investigated using multiple logistic regression analysis (PROC LOGISTIC of SAS). This showed which plant traits (independent terms) mediated AGM herbivory (dependent term). Tree height was measured 3 months after AGM damage assessment, but defoliation by AGM is not considered to have altered tree height significantly. The AGM damage observed in this trial was in the mid-low canopy of trees and other studies have shown that any physiological effects on height would require a greater loss of biomass (>50%; Pinkard *et al.* 2006b), particularly in this short time period.

5.3.4.2 Fenced subset

The 210 tree subset was used for a detailed assessment of the effects of browsing damage on tree traits using a linear mixed model (PROC GLIMMIX of SAS Institute

Inc. 2002-2008 for binary data, and PROC MIXED of SAS for continuous data). Fixed effects in the model were Browse score of the family within the high-bowsed area (none, intermediate and high) and the area (with either low- or high-levels of browsing) in which plants were positioned, and their interaction. Random effects were *replicate* within *area*, and *family* (ignoring race). Foliage physiochemical response to marsupial browsing was investigated by analysing the reflectance wavelength dataset from NIR scans. The dataset was transformed using multi-scatter correction and reduced to 20 principal components using Principal Components Analysis (The Unscrambler 10.1 of Camo Software 2011; the first two principal components explained 52% and 22% of variation respectively). All 20 principal components were analysed to test for the differences between browsing treatments (zero, intermediate and high rate of defoliation) within low and high browsed areas using a single factor MANOVA undertaken with PROC DISCRIM of SAS.

5.4 Results

Across the full trial, tree genetic effects had no significant effect on the pattern of marsupial browsing, with spatial components due to replicates and rows within replicates being more important (see Supplementary material Appendix 2 for detailed results and Table S3). The spatial map of marsupial browsing (Fig. 5.1B) shows a greater proportion of browsed trees at the northern edge of the trial, which is in closest proximity to native forest and more distant from a sealed road running parallel to the southern boundary of the trial. An internal patch of high browsing occurred on the edge of replicate 11, where a large pile of logs and stumps is positioned, and also provided a refuge for marsupials. The spatial map of trees damaged by AGM is the reverse, with greater damage on trees further from areas likely to be refuges for browsing marsupials (data not shown).

5.4.1 Direct effects of marsupial browsing in the fenced and unfenced areas

There were clear direct impacts of marsupial browsing on phenotypic traits subsequently assessed from the *E. globulus* trees (Table 5.2). However, significant marsupial browsing by fence interaction effects for the majority of traits indicate the effect of browsing differed depending on whether trees received a single browsing

event (fenced) or repeated browsing (unfenced). While spatial effects are confounded in the difference between fenced and unfenced areas, all traits exhibit similar trends between the two areas except the effects of browsing were exacerbated in the unfenced areas where browsing continued after assessment (Fig. 5.2A-H).

Mortality within the fenced area at year 1 (Fig. 5.2A) and year 4 (Fig. 5.2B) significantly increased when plants were defoliated in excess of 62.5% with the majority of plant loss occurring within the first year. Survival of the unfenced plants however, showed a greater response to lower damage (37.5%). As expected, height decreased with increased browsing and trees that received repeated browsing were shorter than trees that suffered the single browsing event (Fig. 5.2C). One year post browsing, height of the trees in the fenced area declined by 19% following severe browsing (87.5%). In contrast, the unprotected trees suffered a substantial decrease of 51% at this browse level, mainly due to the continual exposure to browsers.

Basal area of the surviving trees showed a similar response to browsing damage, with a relatively small decrease following severe defoliation (87.5%) in the fenced trees at both assessments. Basal area at 1.7 years decreased by 22% (figure not shown), and 14% at 4 years post browsing (Fig. 5.2D). Tree basal area in the unfenced area was reduced by a considerable 54% and 36%, at 1.7 and 4 years respectively. The proportion of trees with multiple stems showed a similar pattern in year 1 (Fig. 5.2E) and 4 (figure not shown) post marsupial browsing. The incidence of multiple stems increased with browsing severity but declined following extreme defoliation (87.5%). The marked decrease in the proportion of plants with multiple stems in the highest browsing class may reflect mortality of heavily browsed plants which would otherwise develop multiple stems. There were significant impacts of marsupial browsing on vegetative phase change, with a decline in the proportion of trees that had transitioned to adult leaves in the unfenced area (Fig. 5.2F). The proportion of flower buds was affected by marsupial browsing, with a decrease in flower buds in plants defoliated greater than 62.5% and 37.5% in the fenced area and unfenced areas, respectively (Fig. 5.2G). Stem straightness did deteriorate with browsing, with a marked deviation in response to severe damage of 87.5% defoliation, but there was no difference in the response of fenced and unfenced trees (figure not shown).

Table 5.2. The effects of marsupial browsing and its interaction with fencing (protected or unprotected plants) and race general combining ability (GCA) on *E. globulus* fitness surrogate traits and subsequent organism damage, up to four years post damage. While a more complete mixed model was fitted (see methods), only the key fixed terms involving marsupial browsing have been tabulated. The trees in the diallel included in the analysis were from areas that were fenced (n=2658) and unfenced (n=1776) following the initial browsing event just after planting. The table shows the Walds F-statistic and its probability level, with significant effects in bold: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

			Direct effects											Indirect effects
	Ndf	Ddf	Mortality [a]		Height	Basal area		Multiple stems [a]			Adult foliage [a]	Flower buds [a]	Stem straightness [a]	AGM [a]
Year post browsing			1	4	1	1.7	4	1	1.7	4	1.7	3	4	0.7
Marsupial browse	4	3724-4345	**71.8*** **	**52.2*** **	**81.2*** **	**46.3*** **	**28.4*** **	**12.5*** **	**7.7*** **	**5.8*** **	**8.2*** **	**4.0** **	**4.5*** **	**20.5*** **
Fenced	1	14 [b]	**17.9*** **	**25.1*** **	**74.6*** **	**50.2*** **	**28.4*** **	0.0	3.9	0.4	**27.7*** **	**6.0***	**11.5** **	**375.8*** **
Marsupial browse x fenced	4	3550-4345	**6.8*** **	**4.1** **	**18.1*** **	**14.3*** **	**11.2*** **	**5.7*** **	2.2	**2.7***	1.2	**3.4** **	2.1	2.3
Marsupial browse x race GCA	8	3780-4345	1.0	0.8	1.9	**2.1***	1.7	0.5	1.5	1.7	0.4	0.5	0.7	0.7

Ndf = numerator degrees of freedom for fixed effects.
Ddf = denominator degrees of freedom associated with the random error terms used to test the fixed effects; the higher degrees of freedom are associated with reduced terms in binary models.
AGM = Autumn gum moth
[a] Binary trait. [b] The convergence of binary traits required the removal of random design features resulting in high Ddf values.

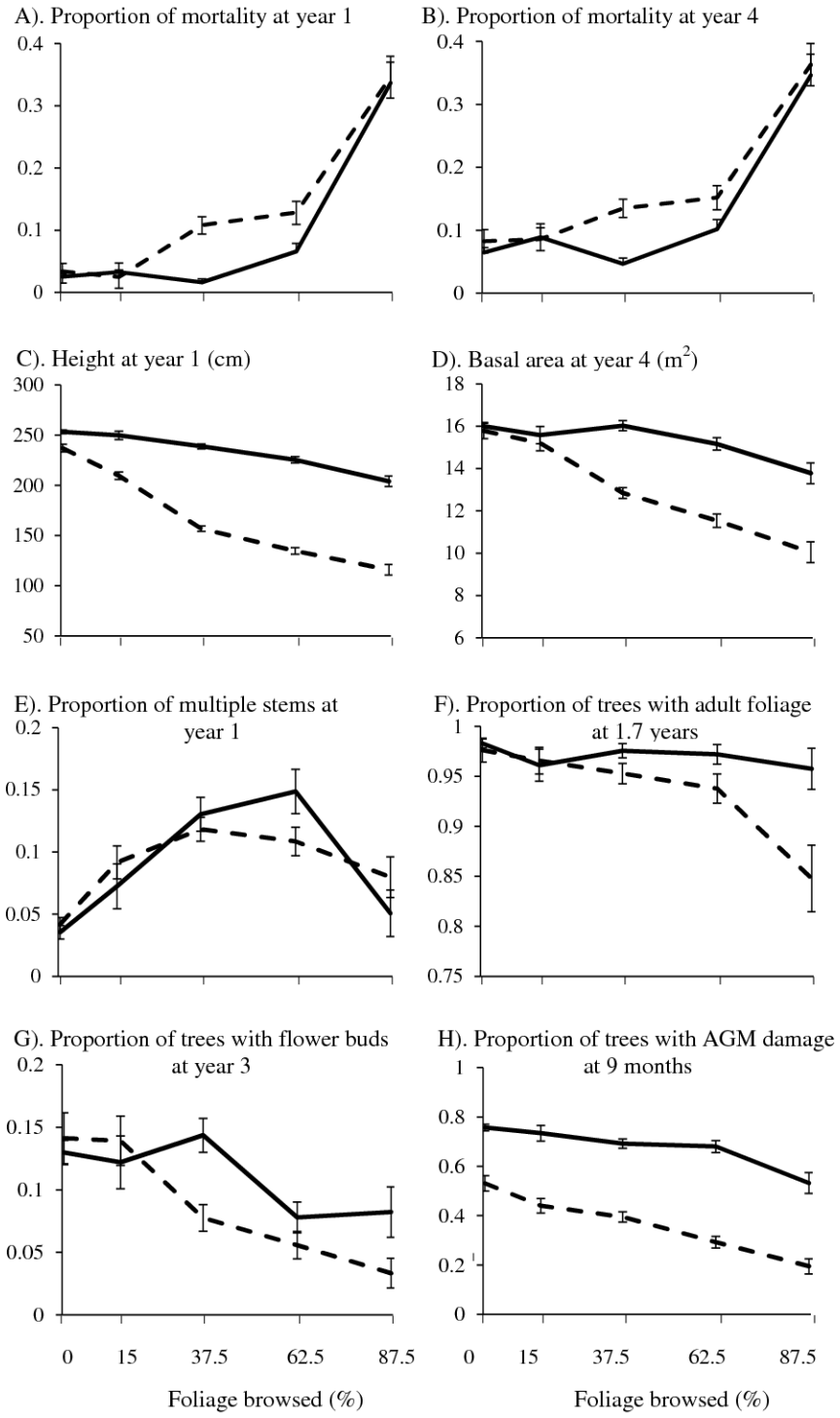

Figure 5.2 (A – H). The effect of marsupial browsing on *E. globulus* fitness and growth traits, and the proportion of trees with AGM damage ± standard errors. Assessed here are plants in the diallel, situated within the fenced (n=2658) and unfenced areas (n=1776). ——— fenced; – – – unfenced.
The x-axis tick marks indicate the browse levels assessed in the study.

The browsing by race GCA interaction was rarely significant (Table 5.2), indicating that the *E. globulus* races did not respond differentially to browsing damage. The only significant racial by browsing interaction term was for DBH at 1.7 years ($P<0.05$), but this effect dissipates the following year. There were no significant three-way interactions ($P>0.05$) between marsupial browse, fence and racial GCA effects. This indicates that the effect of fencing on the response to browsing was similar in plants from the three races.

Fenced subset

Analysis of the 210 tree subset within the fenced area showed a significant browse level by area interaction on tree shape (tree height/width), where browsed plants in the high browsed area responded differently than their unbrowsed pairs in the low browsed area ($F_{2,93}=5.01$, $P=0.009$). Browsed trees in the high browsed area were wider relative to their height compared to corresponding unbrowsed trees of the same families growing in the low browsed area, whereas there was no significant difference between unbrowsed plants in both areas. The effect of browsing on plant height ($F_{2,95}=2.44$, $P=0.09$), width ($F_{2,95}=0.87$, $P=0.42$), basal area ($F_{2,95}=2.45$, $P=0.09$), and number of stems ($F_{2,80}=0.20$, $P=0.82$) was not detected in this subset of plants.

The spectral physicochemical profile of the sampled leaves revealed no difference between plants within the two areas of varying browsing intensities. This indicates that damaged plants did not exhibit differential foliar properties to undamaged plants 7 months after the browse event. Discriminant analysis using 6 categorical groups based on browse treatment within areas showed clear differences between the sampling areas (areas of low- versus high-levels of browsing; $P<0.01$). Plants that received zero, intermediate and high damage within each area were not different from each other in regards to the physicochemical profile. There was no evidence to show that physicochemical differences across areas led to the differential spatial herbivory, as there was no relationship between browsing and physicochemical profile within an area. The differences in spectral physicochemical profile between the two areas confound environmental effects and sampling time (the two areas were sampled at two different times on the same day) effect.

5.4.2 Indirect effects of marsupial browsing in the fenced area

Autumn gum moth (AGM) was present on 70% of the trees one year post browsing, but damage did not exceed 49% defoliation. The presence of AGM damage was linked to marsupial browsing severity (P<.001; Table 5.2), with declining presence on the more heavily browsed plants (Fig. 5.2H). The plants in the unfenced area exhibited similar trends but with lower proportion of AGM at each browse category score (marsupial browse x fenced interaction, P= 0.056; Table 5.2). There was no evidence that the indirect effects of browsing were influenced by focal plant genetics (marsupial browse x race GCA, P=0.7; Table 5.2). When height at year one was included as a covariate in the analysis, the indirect effect of browsing on AGM was reduced substantially and F values indicate that the vast majority of the variation attributed to browsing is accounted for by height (height $F_{1,3998}$=720, P<0.001; browse category $F_{4,3998}$=2.51, P=0.040 compared without the height covariate $F_{4,4000}$ =19.2, P<0.001). Multiple logistic regression analysis run on both the fenced and unfenced trees showed that plant height explained 15.2% and 15.4% of variation respectively (P<0.001), in AGM presence 9 months post browsing. This strong relationship between AGM and plant height suggests that the small genetic effect on AGM is a result of the genetic basis to variation in plant height (Supplementary material Appendix 2 Table S3). The variation in the number of stems also significantly influences AGM presence (P<.001), although alone, it contributes less than 1% to the variation in both fenced and unfenced areas.

5.5 Discussion

This study demonstrates significant impacts of marsupial herbivore damage on plant fitness and morphology traits, and evidence of plant mediated indirect effects between two herbivores in a eucalypt system. Marsupial induced heterogeneity in tree height was important in determining the subsequent infestation of an invertebrate herbivore in this study. Such indirect effects have the potential to influence biotic community structure on a foundation species host-plant, and the evolutionary interactions that occur between organisms and the host-plant themselves (Utsumi 2011, 2013; Walsh 2013).

5.5.1 Spatial and genetic effects on browsing patterns and growth

Spatial effects at a local scale were more important in determining both marsupial browsing and AGM damage patterns in this field trial compared to plant genetic effects. Uneven patterns in marsupial browsing damage may relate to foraging behaviour influenced by proximity to adjacent shelter vegetation (Bulinski and McArthur 2003), where animals maintain a regular balance between vigilance and feeding (e.g. in eucalypt systems, Nersesian *et al.* 2012; and in other systems, van Beest *et al.* 2013). Significant row within replicate effects suggest opportunistic foraging behaviour by both herbivores, with marsupials simply moving down a row of trees, as observed by Marsh (1998), and patterns in AGM damage reflect this in their preference for the larger unbrowsed trees. However, different assessors scored this trial and while regular scoring calibration was carried out during the day, assessors scored the trial by row, and hence, an assessor effect cannot be dismissed (Dutkowski *et al.* 2006).

The lack of genetic basis to browsing in this trial is in contrast to numerous other studies that have reported a clear genetic basis to marsupial browsing and AGM oviposition on *E. globulus* (Floyd *et al.* 2002; Jones *et al.* 2002; O'Reilly-Wapstra *et al.* 2002; Rapley *et al.* 2004b). The obvious discrepancy between this and other studies likely reflects the limited germplasm (three races) used in this current study. Previous work indicates that these three races exhibit above average resistance to marsupial browsing (O'Reilly-Wapstra *et al.* 2002) and the inclusion of more races across the full geographic range of *E. globulus* (Dutkowski and Potts 1999) may have revealed a genetic basis to both marsupial (O'Reilly-Wapstra et al. 2002) and AGM (Farrow *et al.* 1994) browsing preferences.

5.5.2 Direct effects of marsupial browsing

In the current study, marsupial browsing directly impacted functional traits (proportion of multiple stems, tree shape, and stem straightness) and fitness traits (mortality, growth, timing of phase change and flower production) of plants, but the magnitude of the effect depended on the level and duration of the browsing. Within the unprotected/unfenced parts of the trial, repeated browsing magnified the effects on

E. globulus survival, growth and reproductive fitness. Whilst we cannot separate spatial effects from the fenced and unfenced comparisons, the result for the unfenced trees is what we would expect in response to exposure to multiple browsing events. For example, multiple browsing on eucalypt seedlings has been shown to lead to longer term depression in growth (Di Stefano 2003; Pinkard *et al.* 2006a), increased mortality and growth rate (Bulinski and McArthur 1999), and lower reproductive output (O'Reilly-Wapstra *et al.* 2012). Despite these impacts, seedlings within the protected/fenced areas that suffered a single browsing event exhibited a remarkable ability to survive and recover. Results here suggest that if browsing were kept to a minimum (under 37.5%) during the first 12 months, trees can recover without significant consequence on functional traits or fitness surrogates. Within the fenced area there is also evidence that trees can recover from even the most severe browsing damage. Growth traits of the surviving plants in the fenced area demonstrate this, with significant, but relatively small, reductions occurring between trees subject to zero and the most severe damage. In fact these reductions decreased over the 4 year trial assessment period, indicating that after a number of years of growth, it is likely that even the most severely damaged trees that survive will achieve full recovery. This propensity of *E. globulus* to re-grow after defoliation demonstrates a degree of tolerance to defoliation (Eyles *et al.* 2009) which is a well-recognised strategy that plants use in addition to host resistance to reduce the negative impacts of herbivory (Strauss and Agrawal 1999; Tiffin 2000; Muola *et al.* 2010).

Delayed vegetative phase change was observed in response to increased marsupial browsing of *E. globulus* plants. Heteroblastic plants can exhibit distinct physical and chemical characteristics between their juvenile and adult foliage (O'Reilly-Wapstra *et al.* 2007), and these differences have the potential to drive herbivore populations and damage patterns (e.g. Brennan *et al.* 2001; Loney *et al.* 2006). Delayed phase change as a result of marsupial damage may therefore have transient flow-on consequences for associated arthropod communities that specialise on either juvenile or adult leaves (de Little *et al.* 2008), and this type of indirect interaction presents an important avenue for future plant-herbivore studies.

The increased proportion of multiple stems and change in tree shape following mild marsupial browsing damage (>15%) is a response to the removal of apical buds which releases apical dominance (Bryant *et al.* 1991). Similar responses to marsupial browse damage have been previously shown in eucalypts (Bulinski and McArthur 1999; Close *et al.* 2010) and these changes may influence community structures by altering canopy and ground cover. Multiple stems may also represent a significant loss in production if solid wood is sought from the trees (Close *et al.* 2010) as they increase branching and knotting of tree trunks. Stem straightness is another trait important in solid wood production (Callister *et al.* 2011), and in this study showed a response to extreme marsupial damage four years post browsing, indicating a long-term impact of browsing on this trait, that is exacerbated by repeated browsing.

A clear lack of difference in the physicochemical profile of browsed and unbrowsed plants within an area indicated no induced response to the damage. This is not altogether surprising given that the *E. globulus* trees have aged one year since the initial marsupial browsing event. Since the browsing event, the trees experienced a number of seasons, with environmental variation potentially masking any induced effects on foliage. Previous studies have shown herbivore-induced changes in foliar chemistry led to significant changes in subsequent herbivore performance (e.g. Xiao *et al.* 2012), although to date there has been little evidence of foliar chemical induction in *Eucalyptus*, with the only reported cases exhibiting rapid rather than delayed responses (Rapley *et al.* 2008).

5.5.3 Indirect effects of marsupial browsing

Marsupial browsing in this trial showed clear flow-on consequences to subsequent organisms, with browsed plants being less susceptible to the insect herbivore AGM. While marsupial browsing accounted for a small percentage of the variation in AGM damage, the majority of the effect was attributed to the indirect effects of browsing on tree height, where AGM were attracted to taller trees that were not browsed. Such plant-mediated indirect effects of mammalian herbivores have been shown in other systems (e.g. Lind *et al.* 2012), however to date, studies in eucalypt systems have focused on the indirect effects on subsequent herbivores by other ecological

interferences, such as fire (Steinbauer *et al.* 1998), fungal pathogens (Jones *et al.* 2002) and elevated [CO2] and temperature (Murray *et al.* 2013). To the best of our knowledge, this is the first time the indirect effect of marsupial browsing has been shown in a eucalypt system.

The relationship between marsupial browsing and AGM oviposition is not genetically determined and does not appear to be due to induced physicochemical responses of the plant. This contrasts to previous studies where AGM oviposition preference for taller trees in *E. globulus* (Jones *et al.* 2002) and *E. nitens* (Rapley *et al.* 2009) was explained by proportionally better quality foliage on faster-growing trees. Instead, patterns of AGM in this study suggest that the larger, faster growing undamaged trees provide improved habitat suitability or could reflect differences in tree apparency (Feeny 1976), with taller unbrowsed trees being more apparent to ovipositing females. There was also a small but significant increase of AGM on trees bearing multiple stems, further demonstrating how herbivore induced changes in tree architecture may influence subsequent invertebrate communities (Bailey and Whitham 2006). Alternatively, habitat selection may be driven by environmental conditions such as wind or by protection from predators. Immature stages of AGM are known to have a number of natural predators including spiders, wasps and fly species (Lukacs 1999) and such top-down effects of predators may also be important in determining invertebrate distribution (Kaplan *et al.* 2007).

5.6 Acknowledgements

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Chapter 6:
General Discussion

Two important strategies plants use to counter the negative effects of herbivory are chemical resistance and recovery through vegetative growth. Whilst intraspecific genetic variation in eucalypt chemical defence traits has been explored across different environments (O'Reilly-Wapstra *et al.* 2013b), such variation in eucalypt recovery to defoliation has received little attention. Variations in these components are particularly important for foundation species that show extended genetic effects on the dependent community (Whitham *et al.* 2006; Barbour *et al.* 2009b). Ontogenetic variation also has significant impacts on foliar chemical composition (McArthur *et al.* 2010; Goodger *et al.* 2013a), however few studies have considered the genetic-based ontogenetic effect on the chemical expression in seedling stage eucalypts. The research presented in this thesis (Chapter 2-5) addresses gaps in our knowledge of intraspecific variation in chemical defence and plant recovery from mammal damage in the early growth stages of *Eucalyptus globulus*. The *E. globulus* populations selected for this study exhibited extremes of chemical resistance to mammal browsing (O'Reilly-Wapstra *et al.* 2004). These populations were used to explore intraspecific genetic differences in responses of a number of functional and ecologically significant plant traits across ontogeny and varying defoliation treatments. The work presented in this thesis demonstrates the complexity and dynamics of the different strategies eucalypts use to deal with herbivore interactions. Four general findings emerge from this work (Chapters 2-5), which emphasise the interplay of both dynamic and stable responses in shaping the chemical profile of *E. globulus*.

6.1 Phenotypic changes in eucalypt traits in response to ontogeny and defoliation, with flow-on ecological consequences

The first general finding is the clear responses that occurred in multiple components to defoliation of *E. globulus* seedlings. These included changes in chemistry, physiology and growth traits. The observed changes in chemical composition illustrate the complex and dynamic nature of PSMs in this species. Variation was detected in both early seedling ontogeny (Chapter 2), and in regrowth foliage arising from axillary buds after partial defoliation (involving the removal of apical buds; Chapter 3). Foliar terpenes in consecutive individual leaves demonstrated dynamic changes in ontogenetic trajectories from cotyledon stage through to the fifth leaf pair (Chapter 2). Such changes in chemical composition throughout early seedling establishment highlights the fundamental role of ontogeny in plant defence (Boege 2005b; Barton and Koricheva 2010; Barton 2013), with the potential for it to play a considerable role in the interaction between herbivores, pathogens and their host plant (Lawler *et al.* 1999b; Lawler *et al.* 2000; Eyles *et al.* 2003b; Wiggins *et al.* 2003; Steinbauer *et al.* 2004; O'Reilly-Wapstra *et al.* 2005a; Padovan *et al.* 2014). The rapid and strong responses observed through ontogeny emphasise the importance of accounting for ontogenetic variation in chemical defence studies. Future study in this system would benefit from investigating the functional role that terpenes play in mediating biotic interactions at the concentrations shown in this thesis. This will provide an understanding of the potential of foliar defence in eucalypts.

Simulated browsing and natural mammal browsing both had impacts on *E. globulus* seedlings, resulting in reduced survivorship. The surviving plants, however, exhibited remarkable mechanisms of recovery through vegetative growth. Varying degrees of defoliation elicited immediate physiological, growth and chemical responses. Despite this, many of these responses were shown to be short-lived, even in treatments that involved severe loss of biomass. Effects disappeared in the long-term (20 months to 4 years after browsing damage) with minimal negative impacts evident on important growth traits (Chapter 3-5). To increase our understanding of the ability of *Eucalyptus* to withstand the stresses of herbivory, future studies in this system would benefit from also including root organs. The general results of this thesis, that a

single severe defoliation event does not have longer lasting effects on plant growth, could be due to the fact that only above ground responses have been studied. Compensatory responses to defoliation of eucalypts can involve reduced biomass allocation to coarse roots (Eyles *et al.* 2009), and this may affect the plants ability to deal with future stresses, including recovery from repeated browsing as demonstrated in Chapter 5.

A consistent response to defoliation was noted in short- and long-term foliar chemical responses. Chemical traits in regrowth arising from axillary buds showed initial plastic responses to the defoliation treatment, with important defence compounds indicating elevated levels (assessed at 12 weeks after partial defoliation; Chapter 3). The long-term physicochemical profile (assessed at 9-12 months; Chapters 4 and 5), however, showed no change in foliar chemical expression in response to browsing. Such observed phenotypic changes in response to browsing damage may have significant effects on the associated biotic community. During the recovery period (12 months after damage), variation in growth responses to browsing damage was associated with the subsequent tree use by an invertebrate herbivore in a large-scale pedigreed field trial (Chapter 5). In this trial, marsupial browsing induced changes in tree height were linked to altered tree use by the invertebrate autumn gum moth (*Mnesampela privata*). This provided new evidence of plant mediated indirect effects between two herbivores in a eucalypt system, adding to the growing body of literature documenting trait-mediated indirect interactions driven by mammals. To date, studies in other systems have demonstrated inconsistent patterns, for example Hrabar and Du Toit (2014) showed that elephant (*Loxodonta Africana*) browsing on *Colophospermum mopane* trees reduced oviposition by mopane moths (*Imbrasia belina*). In contrast, Lind *et al.* (2012) showed subsequent herbivory by white-tailed deer (*Odocoileus virginianus*) on *Lindera benzoin* trees by specialist caterpillars (*Papilio Troilus*) was greater on previously browed plants by mammals. The combined studies emphasise the potential of browsing mammals to influence the biotic community dependent on *E. globulus*, which may ultimately influence evolutionary interactions between organisms and the host-plant themselves (Utsumi 2011, 2013; Walsh 2013; O'Reilly-Wapstra *et al.* 2014a).

6.2 Genetic stability in eucalypt responses among populations

The second general finding in this thesis was the genetic stability in many chemical and plant recovery responses amongst divergent populations of *E. globulus*. These divergent populations encompassed the two major molecular lineages in the species and different adaptive races within one of these lineages (Jones *et al.* 2013). This genetic stability was evident not only as stable constitutive differences (O'Reilly-Wapstra *et al.* 2004), but in many cases, populations were stable in the way they changed through ontogeny (Chapter 2) and in recovery after defoliation (Chapters 3 and 4). Specifically, populations exhibited a broadly consistent pattern of change in terpene profile throughout ontogeny, with the different behaviour by the two major terpene groups, mono- and sesquiterpenes, and opposing trajectories of sesquiterpene compounds evident in all populations studied (Chapter 2). The most notable example of consistent constitutive differences between populations that occurred across ontogeny and defoliation treatments were the differences in terpene levels among the Tasmanian populations (St Helens and Blue Gum Hill), where their chemical trajectories rarely differed through ontogeny.

Genetic stability in response to browsing was also detected in numerous recovery traits. These responses occurred despite differences in defoliation severities (i.e. partial and decapitation), and modes of recovery (i.e. axillary buds and basal lignotubers). Conservative responses were detected across populations in a number of transient responses to partial defoliation. These included photosynthetic rate and chlorophyll content in remaining foliage after partial defoliation of juvenile *E. globulus,* as well as growth and chemical changes in regrowth arising from previously dormant axillary buds (Chapter 3). Stable genetic responses at the population level were also observed in the changes in growth and foliar physicochemical traits following resprouting after both plant decapitation (Chapter 4) and after varying levels of natural browsing by mammals (Chapter 5).

A growth response after defoliation is often limited by plant resource allocation (Bond and Midgley 2001). The two *E. globulus* populations selected to investigate recovery responses had marked differences in resource allocation, with one having higher levels of key PSMs (O'Reilly-Wapstra *et al.* 2004), smaller lignotubers and

greater basal stem growth than the other (Whittock *et al.* 2003). A key question in the interaction between eucalypts and their herbivores is does differential allocation of resources affect plant growth response to damage? Given the apparent variation in resource allocation among populations (i.e. defensive chemistry and reserves allocated to dormant buds), the uniformity of growth responses in the surviving plants after defoliation was not expected. This suggests there is little adaptive variation in the growth response among *E. globulus* populations response following defoliation. Further, the long-term growth differences of the two Tasmanian populations are consistent with other studies (Stackpole *et al.* 2010), suggesting this this difference is constitutive, and likely reflects differential resource allocation by *E. globulus* populations adapted to wet and dry environments. Indeed, such adaptation itself may have played an important role in shaping recovery traits in this species.

The work presented in this thesis provides valuable insight into the adaptive capacity of recovery mechanisms following defoliation, and contributes to a growing body of work demonstrating the conservative response of *E. globulus* populations to varying environmental factors. For example, stability in the quantitative chemical expression and holistic physicochemical profile was shown across different field trials (O'Reilly-Wapstra *et al.* 2013a; O'Reilly-Wapstra *et al.* 2013b) and in response to manipulated CO_2 levels (McKiernan *et al.* 2012). In another recent paper, O'Reilly-Wapstra *et al.* (2014a) showed genetic stability among *E. globulus* populations across sites in the patterns in attacks by various pathogen and pest species. These studies provide valuable estimates of genetic effects and predicted responses that are useful when plants are transferred to new environments, such as in managed forests. In an eco-evolutionary context, the stability of genetic effects across environments suggests that evolution of traits may play a role in structuring entire communities and is particularly important in foundation species such as *E. globulus* (Barbour *et al.* 2009c). The stable expression of genetic variation in other systems has however, been unexplored, especially in long-lived trees (Silfver *et al.* 2009). Such studies in other species will help fill currently existing gaps in our understanding of their genetics and evolution.

6.3 Notable patterns of change in some key traits among *E. globulus* populations

Intraspecific genetic variation can provide insight into the potential evolutionary processes affecting plant defence and recovery traits. The third key finding in this thesis was that while many of the response patterns were stable amongst populations, there were several notable exceptions involving ecologically important traits. The first was differences ontogenetic trajectories in foliar terpene expression during early seedling growth (Chapter 2). Variation in the ontogenetic trajectory of terpene content was evident among populations and among families within populations, suggesting that adaptive opportunities exist for changing the levels of terpene content through ontogeny. This adds to a number of recent studies observing intraspecific genetic variation in the ontogenetic expression of terpenes (Holeski *et al.* 2012; Moore *et al.* 2014). Marked differences were evident between the two Tasmanian populations and the Australian mainland population, with ontogenetic trajectories varying in magnitude and direction. This was the case in the expression of total terpenes and many of the individual monoterpenes.

The second important example of clear differences amongst populations involved response to defoliation. Intraspecific genetic variation among *E. globulus* populations was evident in seedling sprouting ability after severe loss of biomass (decapitation; Chapter 4). This suggests that resprouting ability plays an important role in enhancing the survival of *E. globulus* seedlings, i.e. fitness, and is subject to selection from stresses including herbivory and drought (Del Tredici 2001). The mechanistic driver of differential sprouting ability is likely to be related to differences in resource storage components, such as lignotubers and roots. However, the functional link between sprouting and these traits confounds population differences which will need to be removed to obtain stronger evidence of a functional link. For example, a future study should focus on association studies comparing families with different lignotuber development from within a single randomly mating population. Repeated trends across multiple populations would provide strong evidence for a functional link between, for example, lignotuber size and survival following severe defoliation.

6.4 Patterns in chemical expression are linked to biosynthetic origins

The fourth key finding was that many of the chemical responses observed could be linked to their biosynthetic origins. In Chapter 2, individual terpene compounds that shared common patterns in the direction and magnitude of trajectories are linked to their production from common carbocation precursors (Fig. 6.1a,b; Keszei *et al.* 2010). For example, the similar trajectories of compounds 1,8-cineole, limonene and α-terpineol reflect the fact that they originate from the same carbocation precursor (Fig. 6.1a[A]; Keszei *et al.* 2010). Another example is the opposing ontogenetic patterns among sesquiterpene compounds (Fig. 6.1b). The sesquiterpene biochemical pathway was highly active in producing β-caryophyllene and α-humulene (Fig. 6.1b[C]) in the first leaf pair but this activity declined thereafter. The sesquiterpene expression is similar between compounds that share carbocation precursors, i.e. aromadendrene and alloaroadendrene (tricyclic structures; Fig. 6.1b[E]); bicyclogermacrene and α-gurjunene (direct products of terpene synthase; Fig. 6.1b[D]); and between β-caryophyllene and α-humulene (direct products of terpene synthase; Fig. 6.1b[C]; Keszei *et al.* 2010) but these groups have opposing ontogenetic trajectories.

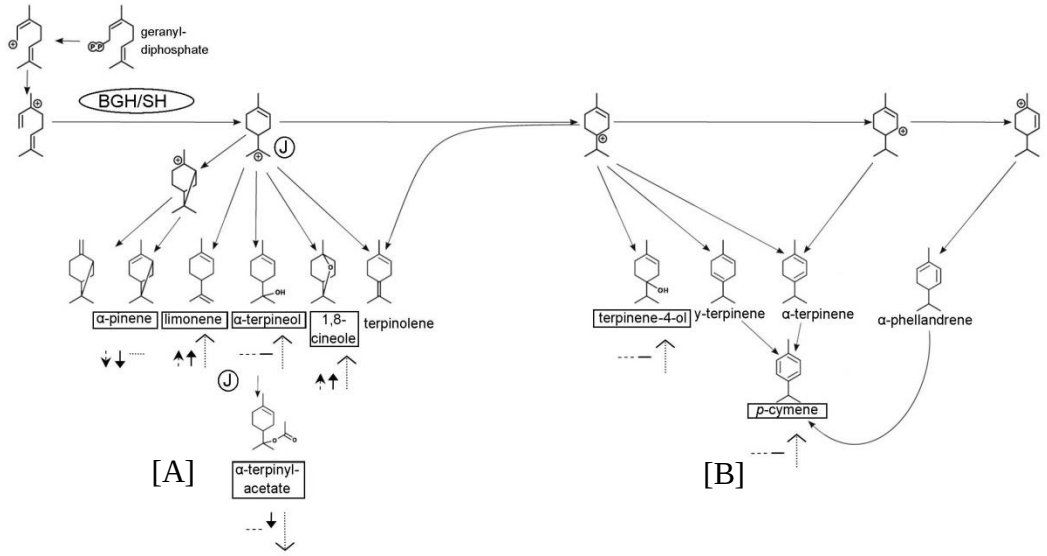
I hypothesised that the observed genetic-based variation in terpene ontogenetic trajectories reflected multiple changes in the regulation of terpene synthase genes (TPS) throughout different terpene biosynthetic pathways (Huber and Bohlmann 2004). The regulation of TPS genes in *Eucalyptus* is capable of generating unique terpene profiles in different parts or at different ontogenetic stages of the plant as required (Külheim *et al.* 2015). In Chapter 2, the key point of difference in Jeeralang plants compared to St Helens and Blue Gum Hill plants appeared to be the behaviour of TPS genes that account for quantitative differences in the trajectory and direction among terpene compounds. Here, changes in the regulation/expression of genes in the Jeeralang population appeared to be occurring at multiple points throughout the terpene biosynthesis pathway (Fig. 6.1a, b). For example, the two Tasmanian populations showed similar ontogenetic patterns in compounds 1,8-cineole, limonene and α-terpineol (Fig. 6.1a[A]; Keszei *et al.* 2010), while those changed more rapidly throughout ontogeny in Jeeralang due to changing activity in a gene

prior to, or at the point of carbocation of the substrate. In another example, the monoterpenes terpineole-4-ol and *p*-cymene are both formed through a different carbocation precursor (Keszei *et al.* 2010) and this may reflect the different ranking of those compounds amongst populations to that of the other monoterpenes. Here, Jeeralang plants expressed the highest content with rapid increases throughout ontogeny, again suggesting that TPS gene(s) in Jeeralang become active prior to or at the point of enzymatic conversion of the intermediate. Terpineole-4-ol is a direct product of terpene synthase whereas the *p*-cymene is synthesised through further modification of primary products in this group (Keszei *et al.* 2010); that difference in synthesis may explain the observed exaggerated population differences in chemical content (Fig. 6.1a[B]).

Patterns of change in chemical composition of regrowth foliage arising from axillary buds after partial defoliation also reflect biosynthetic origins (Chapter 3), including changes in monoterpene compounds as described above. Another example is the lack of significant treatment by crown effect occurring in the only monoterpenes that are products of further modification (*p*-cymene and α-terpinyl acetate). A third example is the common origin of the only two sesquiterpene compounds to change in regrowth (b-caryophyllene and a-humulene). These compounds originate from a common precursor and form earlier in the pathway to the other sesquiterpenes (Keszei *et al.* 2010).

In other chemical compounds, trends were consistent with documented chemical correlations within eucalypt foliage, such as sideroxylonal A and terpene content (Moore *et al.* 2004; Andrew *et al.* 2005). The conservative response of chemical expression in regrowth among *E. globulus* populations suggests that TPS gene regulation occurs early in the synthesis pathway, and chemical production in regrowth may not involve in the same changes in gene expression as the ontogenetic change observed during early seedling ontogeny (cotyledon to leaf node 5, Chapter 2). Alternatively, there may have been stabilisation of the ontogenetic changes by the node on which the defoliation experiment (Chapter 3) was carried out (much later than 5 nodes).

a).



b).

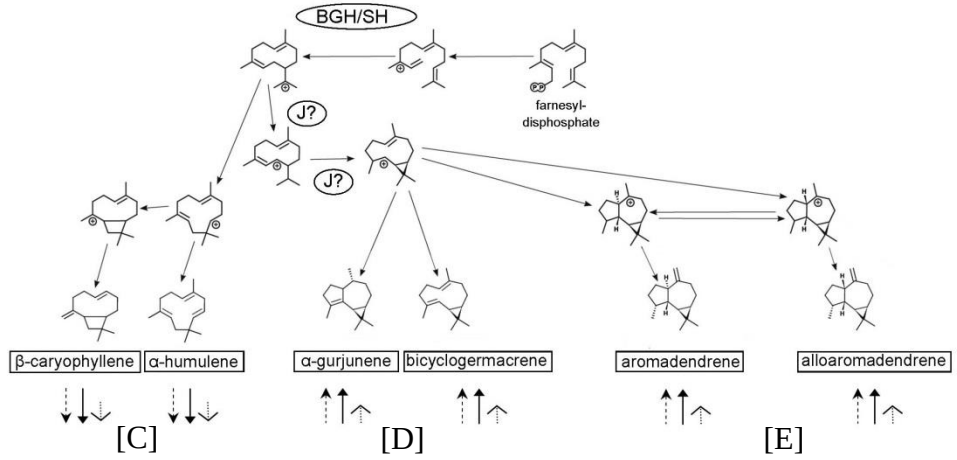
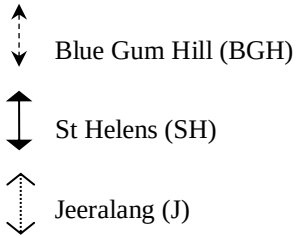


Figure 6.1a,b. The biosynthetic routes for (a) the monoterpenes and (b) the sesquiterpenes, modified from Keszei *et al.* (2010). Common carbocation precursors determine groupings A-E. The compounds described within this thesis (Chapters 2 and 3) are indicated with boxing. Small and large arrows indicate the direction and magnitude of ontogenetic change per compound in each population; dash indicates no ontogenetic change. Circled population names indicate the hypothesised location of gene activity within the pathway.



6.5 Conclusion

I have used a dominant tree species to investigate some important questions of plant-mammal interactions in a eucalypt system. The key strength in using quantitative genetics not only provides important information to better our understanding of natural selection, but provides insight into herbivore preferences, temporal and spatial variation in selection and identification of the type and direction of selection (O'Reilly-Wapstra *et al.* 2012). The findings demonstrated the complexity of different eucalypt strategies that potentially impact on herbivore interactions, and highlight the relevance of implementing a community approach to explore the ecological and evolutionary dynamics between herbivores and their host plants. As discussed in Chapters 2-5 in this thesis, traits exhibiting intraspecific variation in *E. globulus* may provide insight into potential selection pressures from a range of biotic and abiotic factors. The driving forces behind these processes, in this and in other plant systems, however, are in need of further research (O'Reilly-Wapstra *et al.* 2012). Despite the growing literature that is providing support for the role of herbivores as selection agents (Agrawal *et al.* 2010; O'Reilly-Wapstra *et al.* 2010; Barton and Hanley 2013), there is still little evidence for the fundamental question of whether plant enemies are the main drivers of evolutionary diversification, or are they simply responding to underlying genetic variation in their host plants (Futuyma and Agrawal 2009). A key appreciation that comes from the research is the diverse and dynamic nature of the plant chemical profile to which browsers are exposed. Future investigation in this area of research should explore different gene expression using RNA to identify candidate genes in the biosynthetic pathways (Külheim *et al.* 2015) that lead to the phenotypic responses observed. This will identify whether or not parallel chemical responses are linked to the same genes by common gene expression, and where any differences reside, thus allowing a better understanding of the genetic control of the ontogenetic and plastic responses that drive chemical divergence.

The genetic ranking of many quantitative traits examined in plant-herbivore-pathogen studies in eucalypt populations, as demonstrated here and in other studies (McKiernan *et al.* 2012; O'Reilly-Wapstra *et al.* 2013b; O'Reilly-Wapstra *et al.* 2014a), appears to be consistent across environments. In addition to the ecological and evolutionary

importance of the finding, the stability of the relative differences amongst population in many of the traits studied in *E. globulus* has important applied implications for its use for commercial plantations in temperate regions of the world (Myburg *et al.* 2014). For example, the lack of variation among populations in growth after browsing damage, will give growers added confidence that when they artificially select genotypes, such as for increased expression of PSMs to reduce losses from herbivore damage (Miller *et al.* 2009) or improved solid-wood value (Callister *et al.* 2011), plants will demonstrate the same recovery responses where mammal browsing damage does occur.

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Appendices

Appendix 1: Supplementary material for Chapter 2

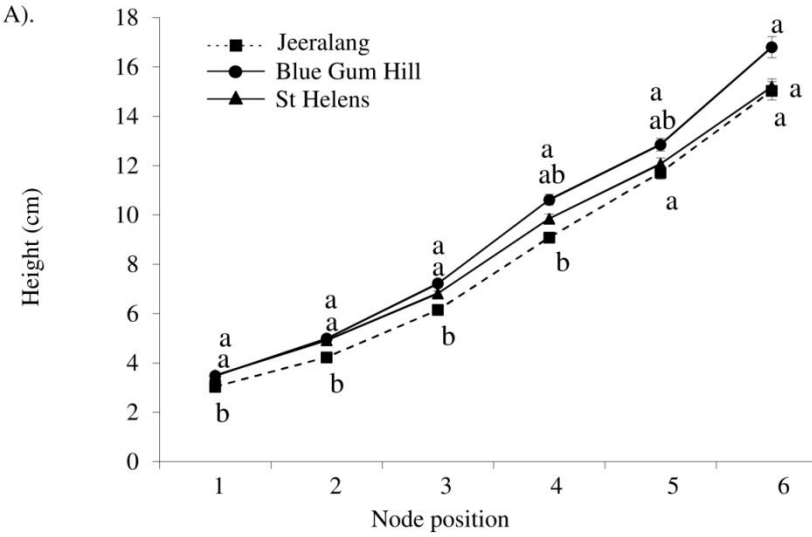
Supplementary Table S1. Significance of random terms in the mixed model analysis used to analyse the terpene attributes at the family level. Total mono- and sesquiterpene content was calculated by the sum of their individual compounds. Significance of effects indicated in bold: *, P < 0.05; **, P < 0.01; ***, P < 0.001; and blank, not significant. '.' indicates insignificant values that could not be estimated as the variance component was at the boundary of the parameter space and thus is considered zero. The random family within population by leaf age terms were rarely significant.

	Family (Population) Z	Family(Population) *Ontogeny Z
Total oil	**3.2 *** **	**2.9 ** **
Total Monoterpenes	**3.3 *** **	**2.8 ** **
Total Sesquiterpenes	**3.3 *** **	**3.7 *** **
Monoterpenes		
1,8-cineole	**3.3 *** **	**3.1 ** **
α-pinene	**3.3 *** **	**1.7 ** *
limonene	**3.2 *** **	**2.6 ** **
α-terpineol	**3.5 *** **	**3.4 *** **
α-terpinyl acetate	**3.5 *** **	.
p-cymene	**3.5 *** **	1.0
terpinene-4-ol	**3.5 *** **	.
2-hydroxy-1,8-cineole	**3.0 ** **	1.0
Sesquiterpenes		
aromadendrene	**3.3 *** **	**3.8 *** **
bicyclogermacrene	**3.2 *** **	**2.5 ** **
alloaromadendrene	**3.2 *** **	**3.4 *** **
α-gurjunene	**3.2 *** **	1.2
β-caryophyllene	**3.4 *** **	**2.5 ** **
α-humulene	**3.4 *** **	**2.7 ** **

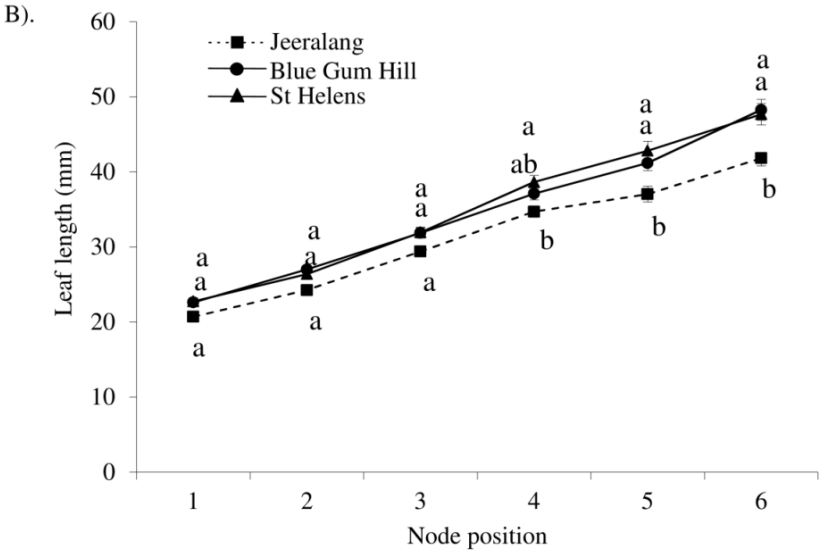
Supplementary Table S2. Summary of mean values expressed in units of mg g^{-1} DM (±SE) of terpene attributes determined for *E. globulus* cotyledonary stage seedlings (n=30 family pools) and the average across all five seedling leaf pairs (n=450). Total mono- and sesquiterpene content was calculated from the sum of their individual compounds. 1,8-Cineole and α-pinene are expressed as milligrams g-1 DM, whilst all other compounds are expressed as equivalents of 1,8-cineole (mg.g-1 DM). Results are presented in order of dominance in seedlings. The fixed effects model analysis of genetic variation in terpene content in cotyledons among three *E. globulus* populations (Jeeralang, Blue Gum Hill and St Helens) are included. Significance of effects indicated in bold: *, P < 0.05; **, P < 0.01.

	Cotyledonary stage seedlings				Average of all true leaves		
				Population effect			
	mean	SE	range	$F_{2,22}$	mean	SE	range
Total terpenes	3.707	0.200	2.43 - 5.68	1.77	13.13	0.23	4.92 - 29.55
Total Monoterpenes	2.403	0.165	1.39 - 4.19	2.39	9.50	0.17	3.86 - 20.01
Total Sesquiterpenes	0.071	0.014	0.02 - 0.30	1.2	0.68	0.03	0.06 - 3.05
Monoterpenes							
1,8-cineole	1.006	0.103	0.23 - 1.96	**4.8***	6.24	0.12	2.19 - 14.39
α-pinene	0.916	0.048	0.64 - 1.52	0.3	2.18	0.04	0.75 - 6.71
limonene	0.100	0.007	0.05 - 0.17	3.2	0.58	0.01	0.14 - 1.55
α-terpineol	0.076	0.023	0.01 - 0.60	2.6	0.20	0.01	0.04 - 1.04
α-terpinyl acetate	0.238	0.025	0.05 - 0.52	0.7	0.13	0.01	0.00 - 0.72
p-cymene	0.057	0.005	0.02 - 0.11	1.8	0.09	0.01	0.01 - 1.69
terpinene-4-ol	0.006	0.001	0.00 - 0.020	0.4	0.05	0.002	0.01 - 0.44
2-hydroxy-1,8-cineole	0.004	0.001	0.00 - 0.020	**7.2****	0.02	0.001	0.00 - 0.07
Sesquiterpenes							
aromadendrene	0.011	0.004	0.00 - 0.08	2	0.33	0.019	0.00 - 1.98
bicyclogermacrene	0.009	0.002	0.00 - 0.04	**5.2***	0.11	0.005	0.00 - 0.94
alloaromadendrene	0.004	0.001	0.00 - 0.03	0.7	0.10	0.005	0.00 - 0.50
α-gurjunene	0.005	0.001	0.00 - 0.05	3	0.05	0.003	0.00 - 0.33
β-caryophyllene	0.026	0.005	0.005 - 0.10	0.3	0.05	0.002	0.00 - 0.32
α-humulene	0.015	0.003	0.003 - 0.06	1.3	0.03	0.001	0.00 - 0.19

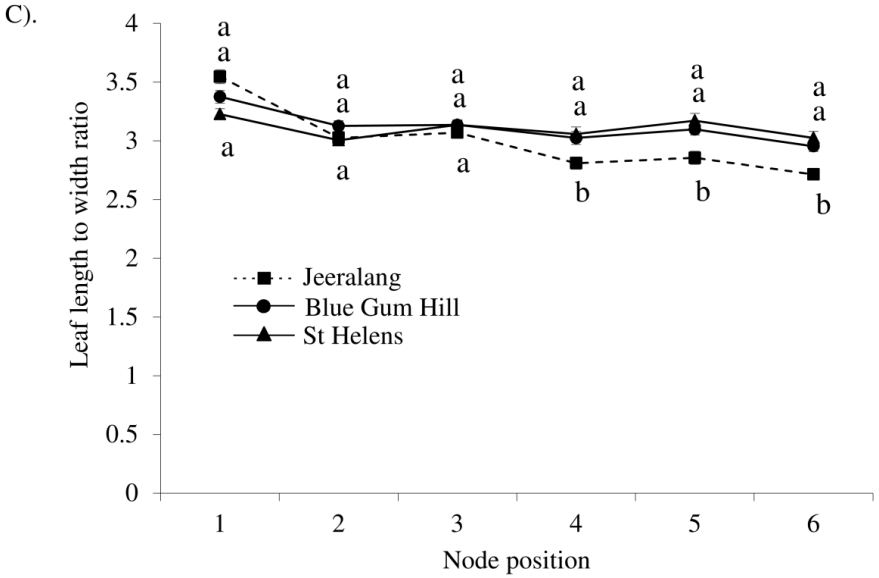
Supplementary Figure S1(A-C). Population variation in *E. globulus* seedling height (A), leaf length (B) and leaf shape (length to width ratio; C) ± standard error at each assessment time at the top node (n=1800). The Australian mainland population is represented by Jeeralang and the two Tasmanian populations are Blue Gum Hill and St Helens. Letters that differ between populations at each time period indicate values that are significantly different (α = 0.05 after Tukey-Kramer adjustment for multiple comparisons).



Note: Seedling height increased with significant population ($F_{2,27}$=8.51, P=0.001) and family (Z=3.2, P<0.001) effects. Height differed depending on the age of the plant when harvested ($F_{5,135}$=1412, P<0.001) with a significant population by age interaction ($F_{10,135}$=2.1, P=0.028). The significant population by harvest day interaction was due to the growth of the population St Helens slowing relative to the Jeeralang and Blue Gum Hill populations across the assessment period.



Note: Leaf length (of the top leaf) increased with significant population ($F_{2,27}$=6.1, P=0.006) and family (Z=3.1, P=0.001) effects, with leaves of the population Jeeralang becoming shorter from node 5. Leaf length differed depending on the age of the plant when harvested ($F_{5,135}$=382.7, P<0.001), with populations responding in a similar manner as the seedlings matured.



Note: Seedling leaf shape (leaf length to width ratio) decreased with the age of the plant when harvested ($F_{5,35}$=31.0, P<0.001) with a significant population by age interaction ($F_{10,35}$=5.8, P<0.001). The significant population by harvest day interaction was due to the leaf shape of Jeeralang seedlings becoming shorter and wider relative to the other populations across the assessment period.

Appendix 2: Supplementary material for Chapter 5

Genetic and non-genetic effects within the diallel

Statistical analysis

Using data from trees in the diallel from the full trial, the significance of genetic and non-genetic effects on early-age traits (e.g. marsupial browsing damage, tree mortality, tree height, and the presence/absence of autumn gum moth damage) were determined by fitting an individual tree mixed model (Dutkowski *et al.* 2002) with ASReml (version 3) (Gilmour *et al.* 2009). The model fitted was:

$$Y = \mu + Rep + Race\ GCA + Race\ SCA + Race\ Maternal + Race\ Reciprocal + Row + Col + Additive + SCA + Maternal + Reciprocal + \varepsilon$$

where Y is the observation for the response traits, μ is the trait mean, Rep is the fixed replicate effect, Race GCA is the fixed race general combining ability and represents the race effect averaged across males and females, Race SCA is the fixed race specific combining ability and is the deviation of the inter-race crosses from their mid-parent value, Race Maternal is the fixed race maternal effect and thus whether races generally perform differently as a male or female, Race Reciprocal is the fixed race reciprocal effect and represents differences between the specific reciprocal crosses between races, *Row* is the random row within replicate effect, *Col* is the random column within replicate effect, *Additive* is the random within-race additive genetic effect, *SCA* is the random within-race specific combining ability effect, *Maternal* is the random within race maternal effect, *Reciprocal* is the random within-race reciprocal effect and ε is the residual. The Race GCA and Race Maternal terms were fitted by amalgamating three variables describing the relative contribution of each race to (i) the pedigree of each plant and (ii) the pedigree of mother of each plant, respectively, using the grouping option (!G) in ASReml. The additive genetic effects were estimated using a numerator relationship matrix defined using a two-generation pedigree file to determine the genetic covariance between relatives. For presence/absence traits (mortality and AGM damage), a binary model with a logit

link function was fitted. The significance of the fixed effects was tested using the incremental Walds F statistic with the denominator degrees of freedom calculated using the default algebraic derivatives algorithm. The significance of the variance components was tested by using the Z-test (Zar 1984). To determine the proportion of observed variation within race due to additive genetic variation after accounting for spatial effects, within race narrow-sense heritability (h^2) and its standard error for each parameter were calculated with ASReml by dividing the additive genetic variance component by the total phenotypic variance (estimated as the sum of the *Additive, SCA, Maternal, Reciprocal and residual variance components*). To avoid biased estimation, random terms were not constrained be greater than zero.

Results

Across the diallel in the full trial, there was no effect of plant genotype on marsupial browsing (Supplementary Table S3). Marsupial browsing patterns were therefore not related to plant genetics, allowing the investigation of the direct and indirect consequence of browsing without the confounding effects of genetics. For the presence of autumn gum moth the race SCA effect (describing the deviation of inter-race crosses from their mid-parent value) was statistically significant, the effect was small compared with spatial factors such as replicate (Supplementary Table S3). There was a significant effect of race (race GCA) on tree mortality, and this was due to a relatively higher survival rate and slower growth of plants originating from the Strzelecki race (data not shown). For one-year height there was a significant effect of race SCA and within race additive genetic and reciprocal variation. Such differential genetic patterns in these traits have been previously shown in these populations (e.g. Dutkowski and Potts 1999; Stackpole *et al.* 2010), however, the early non-maternal reciprocal effect on tree height may be a product of a residual nursery effect that often disappears with age (see Lopez *et al.* 2003), and is no longer evident in basal area at 2 and 4 years post browsing (data not shown). Based on the standard error estimates, the heritability values for organism damage and mortality at year 1 were all effectively zero and that for year 1 height marginally greater than zero.

Supplementary Table S3. Genetic and non-genetic effects on damage by marsupials, mortality, height (1 year post browsing) and the proportion of trees with damage by autumn gum moth (AGM; 9 months post browsing) across the entire trial (diallel only; n=4040 to 4427 depending on trait). The random additive, specific combining ability, maternal effects refer to within race effects. Walds *F* values are presented for fixed effects and *Z* values are presented for the random effects. Significant effects are in bold: *, P < 0.05; **, P < 0.01; and ***, P < 0.001. The model was fitted with random effects unconstrained, meaning some variance component estimates were less than zero.

			Marsupial Browse	Mortality [a]	Height	AGM [a]
Source of variation	*Ndf*	*Ddf*	F/Z	F/Z	F/Z	F/Z
Fixed effects						
Replicate	14	238-299	**24.2*** **	**3.9*** **	**26.7*** **	**18.4*** **
Race general combining ability	2	21-26	1.4	**6.1** **	**3.2**	0.7
Race specific combining ability	3	128-321	0.0	0.4	**6.3*** **	**2.7***
Race maternal	2	30-63	2.6	1.3	1.3	1.4
Race reciprocal	1	225-273	0.0	0.2	0.1	0.2
Random effects						
Row X			**7.7*** **	**3.5*** **	**9.1*** **	**6.2*** **
Column Y			**4.5*** **	1.3	**8.1*** **	2.9**
Additive			1.3	0.5	**2.5** **	0.4
Specific combining ability			0.0	**1.8***	<0	<0
Maternal			<0	0.2	<0	0.7
Reciprocal			1.1	<0	**2.1***	<0
h^2			0.011	0.039	0.064	0.010
h^2 standard error			0.008	0.070	0.025	0.034

Ndf = numerator degrees of freedom for fixed terms
Ddf = demominator degrees of freedom associated with the random error terms used to test the fixed effects
h^2 = within-race narrow sense heritability estimate
[a] Presence/absence trait fitted using a binary model with a logit link function